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  * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 16:04:54 ON 08 MAY 2001

=> fil .bec,canc

COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.15       0.15

FILES 'MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, BIOSIS, EMBASE, HCAPLUS, NTIS,
       ESBIOBASE, BIOTECHNO, CANCERLIT' ENTERED AT 16:05:40 ON 08 MAY 2001
ALL COPYRIGHTS AND RESTRICTIONS APPLY.  SEE HELP USAGETERMS FOR DETAILS.

11 FILES IN THE FILE LIST

=> s src

FILE 'MEDLINE'
L1         9636 SRC

FILE 'SCISEARCH'
L2         9261 SRC

FILE 'LIFESCI'
L3         4116 SRC

FILE 'BIOTECHDS'
L4          131 SRC

FILE 'BIOSIS'
L5         9346 SRC

FILE 'EMBASE'
L6         7419 SRC

FILE 'HCAPLUS'
L7         9935 SRC

FILE 'NTIS'
L8         1910 SRC

FILE 'ESBIOBASE'
L9         4403 SRC

FILE 'BIOTECHNO'
L10        5345 SRC

FILE 'CANCERLIT'
L11        7163 SRC

TOTAL FOR ALL FILES
L12       68665 SRC

=> s activat?

FILE 'MEDLINE'
L13      465277 ACTIVAT?
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FILE 'SCISEARCH'
L14     536107 ACTIVAT?

FILE 'LIFESCI'
L15     163822 ACTIVAT?

FILE 'BIOTECHDS'
L16      16070 ACTIVAT?

FILE 'BIOSIS'
L17     524613 ACTIVAT?

FILE 'EMBASE'
L18     450466 ACTIVAT?

FILE 'HCAPLUS'
L19     900252 ACTIVAT?

FILE 'NTIS'
L20      26312 ACTIVAT?

FILE 'ESBIOBASE'
L21     172456 ACTIVAT?

FILE 'BIOTECHNO'
L22     179372 ACTIVAT?

FILE 'CANCERLIT'
L23     137566 ACTIVAT?

TOTAL FOR ALL FILES
92 L24    3572313 ACTIVAT?

=> s upstream

FILE 'MEDLINE'
L25      29198 UPSTREAM

FILE 'SCISEARCH'
L26      32227 UPSTREAM

FILE 'LIFESCI'
L27      20431 UPSTREAM

FILE 'BIOTECHDS'
L28       2901 UPSTREAM

FILE 'BIOSIS'
L29      33200 UPSTREAM

FILE 'EMBASE'
L30      26179 UPSTREAM

FILE 'HCAPLUS'
L31      45176 UPSTREAM

FILE 'NTIS'
L32       5803 UPSTREAM
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FILE 'ESBIOBASE'
L33      15732 UPSTREAM

FILE 'BIOTECHNO'
L34      22730 UPSTREAM

FILE 'CANCERLIT'
L35       8739 UPSTREAM

TOTAL FOR ALL FILES
L36     242316 UPSTREAM

=> s l12 and l24 and l36

FILE 'MEDLINE'
L37         180 L1 AND L13 AND L25

FILE 'SCISEARCH'
L38         190 L2 AND L14 AND L26

FILE 'LIFESCI'
L39          80 L3 AND L15 AND L27

FILE 'BIOTECHDS'
L40           0 L4 AND L16 AND L28

FILE 'BIOSIS'
L41         148 L5 AND L17 AND L29

FILE 'EMBASE'
L42         145 L6 AND L18 AND L30

FILE 'HCAPLUS'
L43         163 L7 AND L19 AND L31

FILE 'NTIS'
L44           0 L8 AND L20 AND L32

FILE 'ESBIOBASE'
L45         127 L9 AND L21 AND L33

FILE 'BIOTECHNO'
L46         118 L10 AND L22 AND L34

FILE 'CANCERLIT'
L47         130 L11 AND L23 AND L35

TOTAL FOR ALL FILES
L48        1281 L12 AND L24 AND L36

=> s l12 and l24(5a)l36

FILE 'MEDLINE'
          1907 L13(5A)L25
L49         57 L1 AND L13(5A)L25

FILE 'SCISEARCH'
          1969 L14(5A)L26
L50         59 L2 AND L14(5A)L26
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FILE 'LIFESCI'
          1324 L15(5A)L27
L51         28 L3 AND L15(5A)L27

FILE 'BIOTECHDS'
           123 L16(5A)L28
L52          0 L4 AND L16(5A)L28

FILE 'BIOSIS'
          2025 L17(5A)L29
L53         52 L5 AND L17(5A)L29

FILE 'EMBASE'
          1714 L18(5A)L30
L54         47 L6 AND L18(5A)L30

FILE 'HCAPLUS'
          2486 L19(5A)L31
L55         55 L7 AND L19(5A)L31

FILE 'NTIS'
            10 L20(5A)L32
L56          0 L8 AND L20(5A)L32

FILE 'ESBIOBASE'
          1199 L21(5A)L33
L57         43 L9 AND L21(5A)L33

FILE 'BIOTECHNO'
          1443 L22(5A)L34
L58         39 L10 AND L22(5A)L34

FILE 'CANCERLIT'
           747 L23(5A)L35
L59         40 L11 AND L23(5A)L35

TOTAL FOR ALL FILES
L60        420 L12 AND L24(5A) L36


=> s hbv or hbx

FILE 'MEDLINE'
          8955 HBV
           207 HBX
L61       9051 HBV OR HBX

FILE 'SCISEARCH'
          7305 HBV
           258 HBX
L62       7437 HBV OR HBX

FILE 'LIFESCI'
          3583 HBV
           135 HBX
L63       3641 HBV OR HBX

FILE 'BIOTECHDS'
           383 HBV
             6 HBX
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L64          385 HBV OR HBX

FILE 'BIOSIS'
            9425 HBV
             232 HBX
L65         9539 HBV OR HBX

FILE 'EMBASE'
            7841 HBV
             187 HBX
L66         7930 HBV OR HBX

FILE 'HCAPLUS'
            4224 HBV
             468 HBX
L67         4430 HBV OR HBX

FILE 'NTIS'
              73 HBV
              26 HBX
L68           99 HBV OR HBX

FILE 'ESBIOBASE'
            2328 HBV
             118 HBX
L69         2382 HBV OR HBX

FILE 'BIOTECHNO'
            3938 HBV
             151 HBX
L70         4011 HBV OR HBX

FILE 'CANCERLIT'
            3003 HBV
             134 HBX
L71         3059 HBV OR HBX

TOTAL FOR ALL FILES
L72        51964 HBV OR HBX

=> s l12 and l24 and l72

FILE 'MEDLINE'
L73             5 L1 AND L13 AND L61

FILE 'SCISEARCH'
L74             5 L2 AND L14 AND L62

FILE 'LIFESCI'
L75             2 L3 AND L15 AND L63

FILE 'BIOTECHDS'
L76             0 L4 AND L16 AND L64

FILE 'BIOSIS'
L77             3 L5 AND L17 AND L65

FILE 'EMBASE'
L78             3 L6 AND L18 AND L66
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FILE 'HCAPLUS'
L79             6 L7 AND L19 AND L67

FILE 'NTIS'
L80             0 L8 AND L20 AND L68

FILE 'ESBIOBASE'
L81             3 L9 AND L21 AND L69

FILE 'BIOTECHNO'
L82             3 L10 AND L22 AND L70

FILE 'CANCERLIT'
L83             3 L11 AND L23 AND L71

TOTAL FOR ALL FILES
L84            33 L12 AND L24 AND L72

=> s l12 and l72

FILE 'MEDLINE'
L85             7 L1 AND L61

FILE 'SCISEARCH'
L86             7 L2 AND L62

FILE 'LIFESCI'
L87             4 L3 AND L63

FILE 'BIOTECHDS'
L88             0 L4 AND L64

FILE 'BIOSIS'
L89             7 L5 AND L65

FILE 'EMBASE'
L90             5 L6 AND L66

FILE 'HCAPLUS'
L91             9 L7 AND L67

FILE 'NTIS'
L92             0 L8 AND L68

FILE 'ESBIOBASE'
L93             4 L9 AND L69

FILE 'BIOTECHNO'
L94             5 L10 AND L70

FILE 'CANCERLIT'
L95             5 L11 AND L71

TOTAL FOR ALL FILES
L96            53 L12 AND L72

=> s (160 or 196) not 1999-2001/py

FILE 'MEDLINE'
        919855 1999-2001/PY
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L97         41 (L49 OR L85) NOT 1999-2001/PY

FILE 'SCISEARCH'
       2178416 1999-2001/PY
L98         41 (L50 OR L86) NOT 1999-2001/PY

FILE 'LIFESCI'
        192836 1999-2001/PY
L99         20 (L51 OR L87) NOT 1999-2001/PY

FILE 'BIOTECHDS'
         29687 1999-2001/PY
L100         0 (L52 OR L88) NOT 1999-2001/PY

FILE 'BIOSIS'
       1141649 1999-2001/PY
L101        39 (L53 OR L89) NOT 1999-2001/PY

FILE 'EMBASE'
        975727 1999-2001/PY
L102        35 (L54 OR L90) NOT 1999-2001/PY

FILE 'HCAPLUS'
       2024230 1999-2001/PY
L103        38 (L55 OR L91) NOT 1999-2001/PY

FILE 'NTIS'
         23553 1999-2001/PY
L104         0 (L56 OR L92) NOT 1999-2001/PY

FILE 'ESBIOBASE'
        633952 1999-2001/PY
L105        29 (L57 OR L93) NOT 1999-2001/PY

FILE 'BIOTECHNO'
        269016 1999-2001/PY
L106        27 (L58 OR L94) NOT 1999-2001/PY

FILE 'CANCERLIT'
        174916 1999-2001/PY
L107        29 (L59 OR L95) NOT 1999-2001/PY

TOTAL FOR ALL FILES
L108       299 (L60 OR L96) NOT 1999-2001/PY

=> dup rem l108

PROCESSING COMPLETED FOR L108
L109        54 DUP REM L108 (245 DUPLICATES REMOVED)

=> d tot


L109 ANSWER 1 OF 54  MEDLINE                                  DUPLICATE 1
TI   Differential activation of p70 and p85 S6 kinase isoforms during cardiac
     hypertrophy in the adult mammal.
SO   JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Sep 18) 273 (38) 24610-9.
     Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU   Laser M; Kasi V S; Hamawaki M; Cooper G 4th; Kerr C M; Kuppuswamy D
AN   1998406106     MEDLINE
```

L109 ANSWER 2 OF 54 MEDLINE DUPLICATE 2
TI A novel regulator of p21-activated kinases.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Sep 11) 273 (37) 23633-6.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Bagrodia S; Taylor S J; Jordon K A; Van Aelst L; Cerione R A
AN 1998395067 MEDLINE

L109 ANSWER 3 OF 54 MEDLINE DUPLICATE 3
TI The **src** homology 2-containing inositol phosphatase (SHIP) is the gatekeeper of mast cell degranulation.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 Sep 15) 95 (19) 11330-5.
Journal code: PV3; 7505876. ISSN: 0027-8424.
AU Huber M; Helgason C D; Damen J E; Liu L; Humphries R K; Krystal G
AN 1998409657 MEDLINE

L109 ANSWER 4 OF 54 MEDLINE DUPLICATE 4
TI Targeted disruption of SHIP leads to Steel factor-induced degranulation of mast cells.
SO EMBO JOURNAL, (1998 Dec 15) 17 (24) 7311-9.
Journal code: EMB; 8208664. ISSN: 0261-4189.
AU Huber M; Helgason C D; Scheid M P; Duronio V; Humphries R K; Krystal G
AN 1999077799 MEDLINE

L109 ANSWER 5 OF 54 MEDLINE DUPLICATE 5
TI Repression of Stat3 activity by activation of mitogen-activated protein kinase (MAPK).
SO ONCOGENE, (1998 Dec 17) 17 (24) 3157-67.
Journal code: ONC; 8711562. ISSN: 0950-9232.
AU Jain N; Zhang T; Fong S L; Lim C P; Cao X
AN 1999087336 MEDLINE

L109 ANSWER 6 OF 54 MEDLINE DUPLICATE 6
TI Angiotensin II stimulates p21-activated kinase in vascular smooth muscle cells: role in activation of JNK.
SO CIRCULATION RESEARCH, (1998 Jun 29) 82 (12) 1272-8.
Journal code: DAJ; 0047103. ISSN: 0009-7330.
AU Schmitz U; Ishida T; Ishida M; Surapisitchat J; Hasham M I; Pelech S; Berk B C
AN 1998311057 MEDLINE

L109 ANSWER 7 OF 54 MEDLINE DUPLICATE 7
TI Transcriptional activators direct histone acetyltransferase complexes to nucleosomes.
SO NATURE, (1998 Jul 30) 394 (6692) 498-502.
Journal code: NSC; 0410462. ISSN: 0028-0836.
AU Utley R T; Ikeda K; Grant P A; Cote J; Steger D J; Eberharter A; John S; Workman J L
AN 1998361240 MEDLINE

L109 ANSWER 8 OF 54 MEDLINE DUPLICATE 8
TI X-gene product of hepatitis B virus induces apoptosis in liver cells.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Jan 2) 273 (1) 381-5.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Kim H; Lee H; Yun Y
AN 1998079072 MEDLINE

L109 ANSWER 9 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R)
TI T cell activation deficiency associated with an aberrant pattern of

protein tyrosine phosphorylation after CD3 perturbation in Down's syndrome
SO PEDIATRIC RESEARCH, (AUG 1998) Vol. 44, No. 2, pp. 252-258.
Publisher: INT PEDIATRIC RESEARCH FOUNDATION, INC, 351 WEST CAMDEN ST, BALTIMORE, MD 21201-2436.
ISSN: 0031-3998.
AU Scotese I; Gaetaniello L; Matarese G; Lecora M; Racioppi L; Pignata C (Reprint)
AN 1998:589092 SCISEARCH

L109 ANSWER 10 OF 54 MEDLINE DUPLICATE 9
TI Repression of mitogen-activated protein kinases ERK1/ERK2 activity by a protein tyrosine phosphatase in rat fibroblasts transformed by upstream oncoproteins.
SO JOURNAL OF CELLULAR PHYSIOLOGY, (1998 Jan) 174 (1) 35-47.
Journal code: HNB; 0050222. ISSN: 0021-9541.
AU Gopalbhai K; Meloche S
AN 1998059303 MEDLINE

L109 ANSWER 11 OF 54 MEDLINE DUPLICATE 10
TI Activation of extracellular signal-regulated kinases (ERK1/2) by angiotensin II is dependent on c-**Src** in vascular smooth muscle cells.
SO CIRCULATION RESEARCH, (1998 Jan 9-23) 82 (1) 7-12.
Journal code: DAJ; 0047103. ISSN: 0009-7330.
AU Ishida M; Ishida T; Thomas S M; Berk B C
AN 1998101649 MEDLINE

L109 ANSWER 12 OF 54 HCAPLUS COPYRIGHT 2001 ACS
TI Activation of c-**src** by the hepatitis B virus (**HBx**) protein is essential for ras activation, cell cycle progression and viral replication
SO (1997) 219 pp. Avail.: UMI, Order No. DA9731409
From: Diss. Abstr. Int., B 1997, 58(4), 1696
AU Klein, Nicola Penny
AN 1997:662018 HCAPLUS
DN 127:258578

L109 ANSWER 13 OF 54 MEDLINE DUPLICATE 11
TI Heat shock activates c-**Src** tyrosine kinases and phosphatidylinositol 3-kinase in NIH3T3 fibroblasts.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 Dec 5) 272 (49) 31196-202.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Lin R Z; Hu Z W; Chin J H; Hoffman B B
AN 1998049596 MEDLINE

L109 ANSWER 14 OF 54 MEDLINE
TI 12-O-Tetradecanoylphorbol-13-acetate activates the Ras/extracellular signal-regulated kinase (ERK) signaling pathway upstream of SOS involving serine phosphorylation of Shc in NIH3T3 cells.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 Dec 5) 272 (49) 30599-602.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU El-Shemerly M Y; Besser D; Nagasawa M; Nagamine Y
AN 1998049512 MEDLINE

L109 ANSWER 15 OF 54 MEDLINE DUPLICATE 12
TI Syk and Fyn are required by mouse megakaryocytes for the rise in intracellular calcium induced by a collagen-related peptide.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 Oct 31) 272 (44) 27539-42.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Melford S K; Turner M; Briddon S J; Tybulewicz V L; Watson S P

AN 1998010581 MEDLINE

L109 ANSWER 16 OF 54 MEDLINE DUPLICATE 13
TI A critical role of Lyn and Fyn for B cell responses to CD38 ligation and interleukin 5.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 Sep 16) 94 (19) 10307-12.
Journal code: PV3; 7505876. ISSN: 0027-8424.
AU Yasue T; Nishizumi H; Aizawa S; Yamamoto T; Miyake K; Mizoguchi C; Uehara S; Kikuchi Y; Takatsu K
AN 97439860 MEDLINE

L109 ANSWER 17 OF 54 MEDLINE DUPLICATE 14
TI Activation of **Src** family kinases by hepatitis B virus **HBx** protein and coupled signaling to Ras.
SO MOLECULAR AND CELLULAR BIOLOGY, (1997 Nov) 17 (11) 6427-36.
Journal code: NGY; 8109087. ISSN: 0270-7306.
AU Klein N P; Schneider R J
AN 1998001570 MEDLINE

L109 ANSWER 18 OF 54 MEDLINE DUPLICATE 15
TI Increased enzymatic activity of the T-cell antigen receptor-associated fyn protein tyrosine kinase in asymptomatic patients infected with the human immunodeficiency virus.
SO BLOOD, (1997 Nov 1) 90 (9) 3603-12.
Journal code: A8G; 7603509. ISSN: 0006-4971.
AU Phipps D J; Yousefi S; Branch D R
AN 1998008125 MEDLINE

L109 ANSWER 19 OF 54 MEDLINE DUPLICATE 16
TI MEK1 mediates a positive feedback on Raf-1 activity independently of Ras and **Src**.
SO ONCOGENE, (1997 Sep 25) 15 (13) 1503-11.
Journal code: ONC; 8711562. ISSN: 0950-9232.
AU Zimmermann S; Rommel C; Ziogas A; Lovric J; Moelling K; Radziwill G
AN 1998026220 MEDLINE

L109 ANSWER 20 OF 54 MEDLINE DUPLICATE 17
TI NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain.
SO EMBO JOURNAL, (1997 Mar 17) 16 (6) 1279-90.
Journal code: EMB; 8208664. ISSN: 0261-4189.
AU Su Y C; Han J; Xu S; Cobb M; Skolnik E Y
AN 97280817 MEDLINE

L109 ANSWER 21 OF 54 MEDLINE DUPLICATE 18
TI mu-Opioids activate tyrosine kinase focal adhesion kinase and regulate cortical cytoskeleton proteins cortactin and vinculin in chick embryonic neurons.
SO JOURNAL OF NEUROSCIENCE RESEARCH, (1997 Nov 1) 50 (3) 391-401.
Journal code: KAC; 7600111. ISSN: 0360-4012.
AU Mangoura D
AN 1998030899 MEDLINE

L109 ANSWER 22 OF 54 MEDLINE DUPLICATE 19
TI Anti-ganglioside monoclonal antibody AA4 selectively inhibits IgE-induced signal transduction pathways in rat basophilic leukemia cells.
SO MOLECULAR IMMUNOLOGY, (1997 Feb) 34 (3) 227-35.
Journal code: NG1; 7905289. ISSN: 0161-5890.
AU Stephan V; Seibt A; Dukanovic D; Skasa M; Swaim W D; Berenstein E H;

Siraganian R P; Wahn V
AN 97368198 MEDLINE

L109 ANSWER 23 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R) DUPLICATE 20
TI **Src**: More than the sum of its parts
SO TRENDS IN CELL BIOLOGY, (JUN 1997) Vol. 7, No. 6, pp. 215-217.
Publisher: ELSEVIER SCI LTD, THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD, OXON, ENGLAND OX5 1GB.
ISSN: 0962-8924.
AU Shalloway D (Reprint); Taylor S J
AN 97:445877 SCISEARCH

L109 ANSWER 24 OF 54 MEDLINE DUPLICATE 21
TI Mutant of insulin receptor substrate-1 incapable of activating phosphatidylinositol 3-kinase did not mediate insulin-stimulated maturation of Xenopus laevis oocytes.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Nov 8) 271 (45) 28677-81.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Yamamoto-Honda R; Honda Z; Ueki K; Tobe K; Kaburagi Y; Takahashi Y; Tamemoto H; Suzuki T; Itoh K; Akanuma Y; Yazaki Y; Kadowaki T
AN 97067100 MEDLINE

L109 ANSWER 25 OF 54 MEDLINE DUPLICATE 22
TI Negative signaling via FcgammaRIIB1 in B cells blocks phospholipase Cgamma2 tyrosine phosphorylation but not Syk or Lyn activation.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Aug 16) 271 (33) 20182-6.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Sarkar S; Schlottmann K; Cooney D; Coggeshall K M
AN 96355333 MEDLINE

L109 ANSWER 26 OF 54 MEDLINE DUPLICATE 23
TI Activation of the mitogen-activated protein kinase pathway by fMet-leu-Phe in the absence of Lyn and tyrosine phosphorylation of SHC in transfected cells.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 May 31) 271 (22) 13244-9.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Torres M; Ye R D
AN 96278734 MEDLINE

L109 ANSWER 27 OF 54 MEDLINE DUPLICATE 24
TI Mechanical strain induces pp60src activation and translocation to cytoskeleton in fetal rat lung cells.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1996 Mar 22) 271 (12) 7066-71.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Liu M; Qin Y; Liu J; Tanswell A K; Post M
AN 96215140 MEDLINE

L109 ANSWER 28 OF 54 MEDLINE DUPLICATE 25
TI Platelet-derived growth factor-dependent activation of phosphatidylinositol 3-kinase is regulated by receptor binding of SH2-domain-containing proteins which influence Ras activity.
SO MOLECULAR AND CELLULAR BIOLOGY, (1996 Oct) 16 (10) 5905-14.
Journal code: NGY; 8109087. ISSN: 0270-7306.
AU Klinghoffer R A; Duckworth B; Valius M; Cantley L; Kazlauskas A
AN 96413343 MEDLINE

L109 ANSWER 29 OF 54 MEDLINE
TI The SH3-domain protein Beml coordinates mitogen-activated protein kinase cascade activation with cell cycle control in Saccharomyces cerevisiae.
SO MOLECULAR AND CELLULAR BIOLOGY, (1996 Aug) 16 (8) 4095-106.

Journal code: NGY; 8109087. ISSN: 0270-7306.
AU Lyons D M; Mahanty S K; Choi K Y; Manandhar M; Elion E A
AN 96315634 MEDLINE

L109 ANSWER 30 OF 54 MEDLINE DUPLICATE 26
TI Signaling by ectopically expressed Drosophila Src64 requires the protein-tyrosine phosphatase corkscrew and the adapter downstream of receptor kinases.
SO CELL GROWTH AND DIFFERENTIATION, (1996 Nov) 7 (11) 1435-41.
Journal code: AYH; 9100024. ISSN: 1044-9523.
AU Cooper J A; Simon M A; Kussick S J
AN 97084048 MEDLINE

L109 ANSWER 31 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R)
TI MULTIPLE REQUIREMENTS FOR SHPTP2 IN EPIDERMAL GROWTH FACTOR-MEDIATED CELL-CYCLE PROGRESSION
SO MOLECULAR AND CELLULAR BIOLOGY, (MAR 1996) Vol. 16, No. 3, pp. 1189-1202. ISSN: 0270-7306.
AU BENNETT A M; HAUSDORFF S F; OREILLY A M; FREEMAN R M; NEEL B G (Reprint)
AN 96:155086 SCISEARCH

L109 ANSWER 32 OF 54 MEDLINE DUPLICATE 27
TI Tyrosine kinases are required for catecholamine secretion and mitogen-activated protein kinase activation in bovine adrenal chromaffin cells.
SO JOURNAL OF NEUROCHEMISTRY, (1996 Mar) 66 (3) 1103-12.
Journal code: JAV; 2985190R. ISSN: 0022-3042.
AU Cox M E; Ely C M; Catling A D; Weber M J; Parsons S J
AN 96365695 MEDLINE

L109 ANSWER 33 OF 54 MEDLINE DUPLICATE 28
TI Protein-tyrosine phosphorylation and p72syk activation in human platelets stimulated with collagen is dependent upon glycoprotein Ia/IIa and actin polymerization.
SO THROMBOSIS AND HAEMOSTASIS, (1996 Apr) 75 (4) 648-54.
Journal code: VQ7; 7608063. ISSN: 0340-6245.
AU Asazuma N; Yatomi Y; Ozaki Y; Qi R; Kuroda K; Satoh K; Kume S
AN 96302781 MEDLINE

L109 ANSWER 34 OF 54 MEDLINE DUPLICATE 29
TI Hypoxia and hypoxia/reoxygenation activate **Src** family tyrosine kinases and p21ras in cultured rat cardiac myocytes.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1996 Sep 13) 226 (2) 530-5.
Journal code: 9Y8; 0372516. ISSN: 0006-291X.
AU Seko Y; Tobe K; Takahashi N; Kaburagi Y; Kadowaki T; Yazaki Y
AN 96400292 MEDLINE

L109 ANSWER 35 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R) DUPLICATE 30
TI HIV ENVELOPE-DIRECTED SIGNALING ABERRANCIES AND CELL-DEATH OF CD4(+) T-CELLS IN THE ABSENCE OF TCR CO-STIMULATION
SO INTERNATIONAL IMMUNOLOGY, (JAN 1996) Vol. 8, No. 1, pp. 65-74. ISSN: 0953-8178.
AU TIAN H; LEMPICKI R; KING L; DONOGHUE E; SAMELSON L E; COHEN D I (Reprint)
AN 96:116376 SCISEARCH

L109 ANSWER 36 OF 54 CANCERLIT
TI The cellular response to ionizing radiation (Meeting abstract).
SO Non-serial, (1995). Neoplastic Transformation in Human Cell Systems in Culture:Mechanisms of Carcinogenesis, S5. Workshop, Chicago, IL, September

7-9, 1995:.
AU Kufe D F; Weichselbaum R R
AN 97615960 CANCERLIT

L109 ANSWER 37 OF 54 MEDLINE DUPLICATE 31
TI Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1995 Mar 31) 270 (13) 7197-203.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Guan K L; Butch E
AN 95221370 MEDLINE

L109 ANSWER 38 OF 54 MEDLINE DUPLICATE 32
TI Mechanisms regulating Raf-1 activity in signal transduction pathways.
SO MOLECULAR REPRODUCTION AND DEVELOPMENT, (1995 Dec) 42 (4) 507-14. Ref: 27
Journal code: AN7; 8903333. ISSN: 1040-452X.
AU Morrison D K
AN 96192971 MEDLINE

L109 ANSWER 39 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R) DUPLICATE 33
TI OVEREXPRESSION OF GAP CAUSES THE DELAY OF NGF-INDUCED NEURONAL DIFFERENTIATION AND THE INHIBITION OF TYROSINE PHOSPHORYLATION OF SNT IN PC12 CELLS
SO JOURNAL OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, (31 JUL 1995) Vol. 28, No. 4, pp. 316-322.
ISSN: 1225-8687.
AU YANG S I (Reprint); KAPLAN D
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L109 ANSWER 40 OF 54 MEDLINE DUPLICATE 34
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L109 ANSWER 43 OF 54 CANCERLIT
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L109 ANSWER 45 OF 54 BIOSIS COPYRIGHT 2001 BIOSIS
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L109 ANSWER 48 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R)
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L109 ANSWER 50 OF 54 BIOSIS COPYRIGHT 2001 BIOSIS DUPLICATE 41

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L109 ANSWER 51 OF 54 LIFESCI COPYRIGHT 2001 CSA
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L109 ANSWER 52 OF 54 MEDLINE DUPLICATE 42
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L109 ANSWER 53 OF 54 MEDLINE DUPLICATE 43
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L109 ANSWER 54 OF 54 CANCERLIT
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ISSN: 0419-4217.
AU Dutta A
AN 90668455 CANCERLIT

=> d ab 8,12-14,17,23,27,34,36,43,49-52

L109 ANSWER 8 OF 54 MEDLINE DUPLICATE 8
AB Hepatitis B virus is a causative agent of hepatocellular carcinoma, and in the course of tumorigenesis, the X-gene product (**HBx**) is known to play important roles. Here, we investigated the transforming potential of **HBx** by conventional focus formation assay in NIH3T3 cells. Cells were cotransfected with the **HBx** expression plasmid along with other oncogenes including Ha-ras, v-**src**, v-myc, v-fos, and E1a. Unexpectedly, the introduction of **HBx** completely abrogated the focus-forming ability of all five tested oncogenes. In addition, the cotransfection of Bcl-2, an apoptosis inhibitor, reversed the **HBx** -mediated inhibition of focus formation, suggesting that the observed repression of focus formation by **HBx** is through the induction of apoptosis. Next, to test unequivocally whether **HBx** induces apoptosis in liver cells, we established stable Chang liver cell lines expressing **HBx** under the control of a tetracycline-inducible promoter. Induction of **HBx** in these cells in the presence of 1% calf serum resulted in typical apoptosis phenomena such as DNA

fragmentation, nuclear condensation, and fragmentation. Based on these results, we propose that **HBx** sensitizes liver cells to apoptosis upon hepatitis B virus infection, contributing to the development of hepatitis and the subsequent generation of hepatocellular carcinoma.

L109 ANSWER 12 OF 54 HCAPLUS COPYRIGHT 2001 ACS
AB Unavailable

L109 ANSWER 13 OF 54 MEDLINE DUPLICATE 11
AB There is increasing evidence that cellular responses to stress are in part regulated by protein kinases, although specific mechanisms are not well defined. The purpose of these experiments was to investigate potential **upstream** signaling events **activated** during heat shock in NIH3T3 fibroblasts. Experiments were designed to ask whether heat shock activates p60 c-**Src** tyrosine kinase or phosphatidylinositol 3-kinase (PI 3-kinase). Using in vitro protein kinase activity assays, it was demonstrated that heat shock stimulates c-**Src** and PI 3-kinase activity in a time-dependent manner. Also, there was increased PI 3-kinase activity in anti-phosphotyrosine and anti-c-**Src** immunoprecipitated immunocomplexes from heated cells. Heat shock activated mitogen-activated protein kinase (MAPK) and p70 S6 kinase (S6K) in these cells. The role of PI 3-kinase in regulating heat shock activation of MAPK and p70 S6K was investigated using wortmannin, a specific pharmacological inhibitor of PI 3-kinase. The results demonstrated that wortmannin inhibited heat shock activation of p70 S6K but only partially inhibited heat activation of MAPK. A dominant negative Raf mutant inhibited activation of MAPK by heat shock but did not inhibit heat shock stimulation of p70 S6K. Genistein, a tyrosine kinase inhibitor, and suramin, a growth factor receptor inhibitor, both inhibited heat shock stimulation of MAPK activity and tyrosine phosphorylation of MAPK. Furthermore, a selective epidermal growth factor receptor (EGFR) inhibitor, tryphostin AG1478, and a dominant negative EGFR mutant also inhibited heat shock activation of MAPK. Heat shock induced EGFR phosphorylation. These results suggest that early upstream signaling events in response to heat stress may involve activation of PI 3-kinase and tyrosine kinases, such as c-**Src**, and a growth factor receptor, such as EGFR; activation of important downstream pathways, such as MAPK and p70 S6K, occur by divergent signaling mechanisms similar to growth factor stimulation.

L109 ANSWER 14 OF 54 MEDLINE
AB We investigated the activation of the Ras/ERK signaling pathway by 12-O-tetradecanoylphorbol-13-acetate (TPA) in NIH3T3 fibroblasts. Interestingly, the activation was suppressed not only by dominant negative Raf-1 but also by dominant negative Ras and SOS. Further analysis revealed that TPA treatment induced, dependently on protein kinase C, the mobility shift of p66(shc) in SDS-polyacrylamide gel electrophoresis, which could be prevented by treatment of the Shc immunoprecipitate with serine/threonine-specific protein phosphatase 1 (PP1) or 2A (PP2A). Phosphoamino acid analysis of Shc showed that unlike growth factor-induced Shc phosphorylation, where Shc is mainly phosphorylated at tyrosine residues, TPA-induced phosphorylation was only at serine residues. Like growth factor-induced Shc phosphorylation, which leads to the association of Shc with Grb2, TPA also induced this association, but, correspondingly to the above results, the TPA-induced association was disrupted by in vitro treatment of the Shc immunoprecipitate with PP1. Taken together, these results suggest that the TPA signal was fed at or **upstream** of Shc to **activate** the Ras/ERK signaling pathway involving serine phosphorylation of Shc.

L109 ANSWER 17 OF 54 MEDLINE DUPLICATE 14

AB The **HBx** protein of hepatitis B virus (**HBV**) is a small transcriptional transactivator that is essential for infection by the mammalian hepadnaviruses and is thought to be a cofactor in **HBV** -mediated liver cancer. **HBx** stimulates signal transduction pathways by acting in the cytoplasm, which accounts for many but not all of its transcriptional activities. Studies have shown that **HBx** protein activates Ras and downstream Ras signaling pathways including Raf, mitogen-activated protein (MAP) kinase kinase kinase (MEK), and MAP kinases. In this study, we investigated the mechanism of activation of Ras by **HBx** because it has been found to be central to the ability of **HBx** protein to stimulate transcription and to release growth arrest in quiescent cells. In contrast to the transient but strong stimulation of Ras typical of autocrine factors, activation of Ras by **HBx** protein was found to be constitutive but moderate. **HBx** induced the association of Ras **upstream activating** proteins Shc, Grb2, and Sos and stimulated GTP loading onto Ras, but without directly participating in complex formation. Instead, **HBx** is shown to stimulate Ras-activating proteins by functioning as an intracellular cytoplasmic activator of the **Src** family of tyrosine kinases, which can signal to Ras. **HBx** protein stimulated c-**Src** and Fyn kinases for a prolonged time. Activation of **Src** is shown to be indispensable for a number of **HBx** activities, including activation of Ras and the Ras-Raf-MAP kinase pathway and stimulation of transcription mediated by transcription factor AP-1. Importantly, **HBx** protein expressed in cultured cells during **HBV** replication is shown to activate the Ras signaling pathway. Mechanisms by which **HBx** protein might activate **Src** kinases are discussed.

L109 ANSWER 23 OF 54 SCISEARCH COPYRIGHT 2001 ISI (R) DUPLICATE 20

AB The **Src** family of protooncoproteins is required for progression through at least two phrases of cell cycle and for some cell-type-specific functions. Recent crystal structures of large fragments of two representatives reveal a compact arrangement of their **Src** -homology 3 (SH3), SH2 and catalytic domains that embodies an unexpected mechanism of regulation. They show that the enzymatic activity of **Src** is controlled by intramolecular associations between the SH2 domain and C-tail and between the SH3 domain and a surprising internal target. The structures highlight a mechanism by which substrates can compete with internal sequences for binding to the SH3 and SH2 domains, thereby stimulating kinase activity. This implies that the distinction between **upstream activators** and downstream effectors will sometimes be ambiguous.

L109 ANSWER 27 OF 54 MEDLINE DUPLICATE 24

AB We have previously shown that mechanical strain-induced fetal rat lung cell proliferation is transduced via the phospholipase C-gamma-protein kinase C pathway. In the present study, we found that protein-tyrosine kinase activity of fetal lung cells increased after a short period of strain, which was accompanied by tyrosine phosphorylation of proteins of approximately 110-130 kDa. Several components of this complex were identified as pp60srcsubstrates. Strain increased pp60src activity in the cytoskeletal fraction, which coincided with a shift in subcellular distribution of pp60src from the Triton-soluble to the cytoskeletal fraction. Strain-induced pp60src translocation did not appear to be mediated via the focal adhesion kinase-paxillin pathway. In contrast, strain increased the association between pp60src and the actin filament-associated protein of 110 kDa. Preincubation of cells with herbimycin A, a tyrosine kinase inhibitor, abolished strain-induced

phospholipase C-gamma1 tyrosine phosphorylation and its coimmunoprecipitation with pp60src. It also inhibited strain-induced DNA synthesis. These results suggest that **activation** of pp60src is an **upstream** event of the phospholipase C-gamma-protein kinase C pathway that may represent an important mechanism by which mechanical perturbations are converted to biological reactions in fetal lung cells.

L109 ANSWER 34 OF 54 MEDLINE DUPLICATE 29
AB We previously reported that both hypoxia and hypoxia followed by reoxygenation (hypoxia/reoxygenation) rapidly and sequentially activate mitogen-activated protein kinase kinase kinase (MAPKKK) activity of Raf-1. This was followed by the sequential activation of MAP kinase kinase (MAPKK). MAP kinases (p42mopk and p44mopk), and S6 kinase (p90rsk). In this study, we demonstrated that both hypoxia and hypoxia/ reoxygenation caused rapid activation of **Src** family tyrosine kinases, p60c-**src** and p59c-fyn, which are **upstream** mediators of MAP kinase **activation**. This was followed by the activation of p21ras. Because **Src** family tyrosine kinases are known to be cell-surface-associated kinases and upstream regulators of p21ras, these results strongly suggested that activation of **Src** family tyrosine kinases plays a key role in triggering intracellular signaling cascades in cardiac myocytes in response to hypoxia and hypoxia/reoxygenation.

L109 ANSWER 36 OF 54 CANCERLIT
AB Eukaryotic cells respond to ionizing radiation (IR) with cell cycle arrest, activation of DNA-repair mechanisms and, in the event of irreparable DNA damage, cell death. While the signaling events that control the cellular responses to IR remain unclear, involvement of nuclear transduction pathways has been supported by the finding that IR activates transcription of c-jun and other early response genes. IR treatment is also associated with activation of the MAP kinase/pp90rsk cascade and increased binding of Jun/AP-1 to the c jun promoter. The recent finding that SAP kinase (JNK) is activated by IR exposure provides support for a stress-associated pathway in the induction of c-jun. We have identified a nuclear protein tyrosine kinase (PTK) which is **activated** by IR and which functions **upstream** to the SEK1/SAP kinase pathway. The nuclear PTK associates with p56/p53lyn and forms a complex with p34cdc2. Since p34cdc2 is phosphorylated on tyrosine in irradiated cells, formation of a nuclear complex with activated PTKs may contribute to IR-induced arrest at the Cdc2-dependent G2 checkpoint. The mechanisms responsible for activation of nuclear PTKs in irradiated cells are unclear. However, IR-induced DNA damage may be an initial signal.

L109 ANSWER 43 OF 54 CANCERLIT
AB Constitutive activity of the v-**Src** protein-tyrosine kinase results in cell transformation and initiates multiple intracellular signalling mechanisms. Okadaic acid (OA) is an inhibitor of the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A). Addition of OA to v-**Src**-transformed BALB/c 3T3 cells reverted them to a flat morphology, increased fibronectin levels in the extracellular matrix, reduced saturation density, and inhibited the formation of colonies in soft agar. The ability of v-**Src**-transformed cells to proliferate in low serum was also inhibited by okadaic acid. These data indicate that OA can inhibit v-**Src**-induced cell transformation. v-**Src** can activate promoters under the control of 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TREs) and serum response elements (SREs). The induction of SRE-mediated gene expression by v-**Src** requires Ras and Raf-1, while the induction of TRE-mediated gene expression by v-

Src requires Ras but is independent of Raf-1. The induction of TRE-mediated gene expression by v-**Src** and v-HaRas increased in the presence of the catalytic subunit of PP2A The induction of SRE-mediated gene expression by v-**Src** and v-HaRas, however, was inhibited by PP2A. PP2A had no effect upon v-Raf-induced SRE-mediated gene expression. These findings implicate serine/threonine phosphatases in v-**Src**-induced cell signalling. They further suggest that PP2A differentially regulates two intracellular signals activated by v-**Src** and v-HaRas, and that the effect of PP2A upon v-**Src** -and v-HaRas-induced SRE-mediated gene expression is **upstream** from Raf-1 **activation**. Thus, serine/threonine phosphatases may play an important role in the transformation of cells by v-**Src**; their activity may either inhibit or potentiate v-**Src**-induced cell signalling depending upon the effect of dephosphorylation on the activity of the substrate protein. (Full text available from University Microfilms International, Ann Arbor, MI, as Order No. AAD94-17468)

L109 ANSWER 49 OF 54 MEDLINE DUPLICATE 40
AB In the current study we sought to elucidate the molecular mechanisms which might contribute to hepatocarcinogenesis in a hepatitis B virus (**HBV**) envelope transgenic mouse model in which chronic hepatocellular injury and inflammation lead to regenerative hyperplasia and eventually to the development of chromosomal abnormalities and hepatocellular carcinoma (HCC), thereby reiterating many of the pathophysiological events that occur prior to the development of HCC in chronic **HBV** infection in humans. We have previously demonstrated that **HBV** envelope gene expression is decreased in regenerating hepatocytes and preneoplastic nodules early in the disease process and that expression of alpha-fetoprotein and the multidrug transporter gene mdr-III is activated in the tumors that develop in this model, but not prior to tumor development. In the current study, we examined the structure and expression of a large panel of dominant acting oncogenes and tumor suppressor genes in the liver at all stages of the disease process in order to determine the extent to which they contribute to hepatocarcinogenesis in these transgenic mice. To our surprise, no changes were observed in the structure or function of any of these genes, many of which are commonly activated in other rodent models of hepatocarcinogenesis but rarely activated in human HCC. These findings suggest that the **HBV** transgenic mouse model is different from most other rodent models of hepatocarcinogenesis and that it may relate more closely to the events involved in **HBV**-induced human hepatocarcinogenesis, where generalized chromosomal abnormalities are common, while structural and functional changes in most of the commonly studied positive-acting oncogenes examined herein are not. Since p53 and RB mutations have recently been reported to be late events in human hepatocarcinogenesis, the structural integrity of the RB locus and the absence of p53 mutations in the **HBV** transgenic mouse model suggest that they may represent a relatively early stage of hepatocellular tumorigenesis and that further manipulation of this model is warranted in order to more fully reproduce the molecular-genetic events that characterize **HBV**-induced HCC in humans.

L109 ANSWER 50 OF 54 BIOSIS COPYRIGHT 2001 BIOSIS DUPLICATE 41
AB Expression of PreS1/PreS2/S gene is regulated by complex interplay between regulatory sequences in the PreS1 and PreS2 promoters and many other positive and negative elements, such as viral enhancers, cis-acting element in the **HBx** gene, cellular transcription factors. Although the exact mechanisms that regulate expression of surface antigen genes are not clear, cell line specificity and subtype specificity were observed by many studies which used adw and ayw subtypes, that are not

common in Korea. The DNA fragments of the PreS1 and PreS2 promoters were amplified from the **HBV** DNA (subtype adr) which were isolated from a Korean HBsAg carrier by polymerase chain reaction (PCR). Oligonucleotides which contains either Hind III or Xba I recognition sites on their 5' ends or 3' ends respectively were generated by oligonucleotide synthesizer and they were used as primers for PCR. After ligating these DNA fragments into chloramphenicol acetyl transferase (CAT) reporter gene, the promoter activities were analyzed by transient transfection assays. The influences of cytokines, oncogenes, tumor suppressor gene, viral transactivators and transcription factor on the activities of PreS1 and PreS2 promoters were screened by CAT assays. While Ras, **Src**, E7, SV40T and AP-2 increased the PreS1 and PreS2 promoter activities, tumor necrosis factor .alpha. was shown to inhibit these promoter activities.

L109 ANSWER 51 OF 54 LIFESCI COPYRIGHT 2001 CSA
AB The expression of nine oncogenes (c-myc, N-myc, N-ras, H-ras, k-ras, abl, fos, **src**, and raf) and two tumor suppressor genes (p53 and RB) were studied by northern blot hybridization in six human hepatocellular carcinoma or hepatoblastoma cell lines (PLC/PRF/5, Hep3B, Hep G2, 2.2.15, HLE, and HLF) and in a human embryonic lung fibroblast cell line (WI-38) to look for differences that might be associated with the presence (PLC/PRF/5, Hep3B, and 2.2.15) or absence (Hep G2, HLE, and HLF) of integrated hepatitis B virus (**HBV**) DNA. The levels of expression of the oncogenes and tumor suppressor genes were unrelated to the presence or absence of integrated **HBV**-DNA. Furthermore, the intensity of expression of these oncogenes was no greater in the 2.2.15 cell line (consisting of Hep G2 cells transfected with hepatitis B virus) than in untransfected Hep G2 cells.

L109 ANSWER 52 OF 54 MEDLINE DUPLICATE 42
AB The expression of nine oncogenes (c-myc, N-myc, N-ras, H-ras, k-ras, abl, fos, **src**, and raf) and two tumor suppressor genes (p53 and RB) were studied by northern blot hybridization in six human hepatocellular carcinoma or hepatoblastoma cell lines (PLC/PRF/5, Hep3B, Hep G2, 2.2.15, HLE, and HLF) and in a human embryonic lung fibroblast cell line (WI-38) to look for differences that might be associated with the presence (PLC/PRF/5, Hep3B, and 2.2.15) or absence (Hep G2, HLE, and HLF) of integrated hepatitis B virus (**HBV**) DNA. The levels of expression of the oncogenes and tumor suppressor genes were unrelated to the presence or absence of integrated **HBV**-DNA. Furthermore, the intensity of expression of these oncogenes was no greater in the 2.2.15 cell line (consisting of Hep G2 cells transfected with hepatitis B virus) than in untransfected Hep G2 cells.

=> log y

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	57.45	57.60

STN INTERNATIONAL LOGOFF AT 16:23:26 ON 08 MAY 2001

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	3175	src	USPAT; US-PGPUB	2001/05/08 12:13
2	L2	479701	activat$8	USPAT; US-PGPUB	2001/05/08 12:20
3	L3	146498	upstream	USPAT; US-PGPUB	2001/05/08 12:20
4	L4	1449	2 near5 3	USPAT; US-PGPUB	2001/05/08 12:21
5	L5	0	4 near5 1	USPAT; US-PGPUB	2001/05/08 12:22
6	L6	5	4 same 1	USPAT; US-PGPUB	2001/05/08 12:22
7	L7	12	1 same 2 same 3	USPAT; US-PGPUB	2001/05/08 13:57
8	L8	133	1 near2 2	USPAT; US-PGPUB	2001/05/08 14:39
9	L9	72	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2001/05/08 14:37
10	L10	1538	hbv or hbx	USPAT; US-PGPUB	2001/05/08 14:37
11	L11	1	9 and 10	USPAT; US-PGPUB	2001/05/08 14:39
12	L12	201	1 near5 2	USPAT; US-PGPUB	2001/05/08 14:39
13	L13	7	10 and 12	USPAT; US-PGPUB	2001/05/08 14:39

	L #	Hits	Search Text	DBs	Time Stamp
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2	L2	479701	activat$8	USPAT; US-PGPUB	2001/05/08 12:20
3	L3	146498	upstream	USPAT; US-PGPUB	2001/05/08 12:20
4	L4	1449	2 near5 3	USPAT; US-PGPUB	2001/05/08 12:21
5	L5	0	4 near5 1	USPAT; US-PGPUB	2001/05/08 12:22
6	L6	5	4 same 1	USPAT; US-PGPUB	2001/05/08 12:22

US-CL-CURRENT: 514/332,514/333 ,546/255 ,546/256 ,546/261 ,546/262 ,546/265 ,546/266

US-PAT-NO: 6184237
DOCUMENT-IDENTIFIER: US 6184237 B1
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: February 6, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/335,514/332 ,514/333 ,546/255 ,546/256 ,546/261 ,546/262 ,546/265 ,546/266

ABSTRACT:
Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, suchas pain and diabetes. The inventin encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermedates useful in such processes.
20 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:
The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducible proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDG, EGF, acidic FDF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antogonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of upstream activators of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 514/252.05,514/253.01 ,514/256 ,514/269 ,514/272 ,514/274
,514/275 ,514/277 ,514/307 ,514/308 ,514/309 ,514/310 ,514/311 ,514/312
,514/313 ,514/314 ,514/332 ,514/334 ,514/336 ,514/337 ,514/339 ,514/340
,514/343 ,544/238 ,544/295 ,544/296 ,544/298 ,544/299 ,544/300 ,544/310
,544/316 ,544/319 ,544/320 ,544/321 ,544/323 ,544/324 ,544/326 ,544/327
,544/328 ,544/330 ,544/331 ,544/333 ,544/364 ,546/139 ,546/140 ,546/141
,546/142 ,546/143 ,546/144 ,546/148 ,546/152 ,546/153 ,546/155 ,546/156
,546/157 ,546/159 ,546/167 ,546/255 ,546/256 ,546/257 ,546/268.1

US-PAT-NO: 6174901
DOCUMENT-IDENTIFIER: US 6174901 B1
TITLE: Substituted pyridine and pyridazine compounds and methods of use
DATE-ISSUED: January 16, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Hwang; Chan-Kou	Boulder	CO	N/A	N/A
Spohr; Ulrike D.	Boulder	CO	N/A	N/A

US-CL-CURRENT: 514/333,514/252.05 ,514/253.01 ,514/256 ,514/269 ,514/272
,514/274 ,514/275 ,514/277 ,514/307 ,514/308 ,514/309 ,514/310 ,514/311
,514/312 ,514/313 ,514/314 ,514/332 ,514/334 ,514/336 ,514/337 ,514/339
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,544/310 ,544/316 ,544/319 ,544/320 ,544/321 ,544/323 ,544/324 ,544/326
,544/327 ,544/328 ,544/330 ,544/331 ,544/333 ,544/364 ,546/139 ,546/140
,546/141 ,546/142 ,546/143 ,546/144 ,546/148 ,546/152 ,546/153 ,546/155
,546/156 ,546/157 ,546/159 ,546/167 ,546/255 ,546/256 ,546/257 ,546/268.1
ABSTRACT:

Selected novel substituted pyridine and pyridazine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as cancer, pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, cancer, pain, diabetes and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.

31 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:

GB 2,306,108, which is incorporated herein by reference in its entirety, describes imidazole derivatives which are Raf kinase antagonists useful in the treatment of cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds may be oncolytics through the antagonism of Raf kinase. It has been reported that antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover, Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of upstream activators of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 424/94.5,435/194

US-PAT-NO: 6040149
DOCUMENT-IDENTIFIER: US 6040149 A
TITLE: Assay for identifying agents which act on the ceramide-activated protein kinase, kinase suppressor of ras, and methods of using said agents
DATE-ISSUED: March 21, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kolesnick; Richard N.	New York	NY	N/A	N/A
Liu; Jun	Boston	MA	N/A	N/A
Zhang; Yuhua	New York	NY	N/A	N/A

US-CL-CURRENT: 435/15,424/94.5 ,435/194
ABSTRACT:

The subject invention provides a purified membrane-bound ceramide-activated protein kinase having an apparent molecular weight of about 110 kD as determined by SDS polyacrylamide gel electrophoresis, which protein kinase is capable of specifically phosphorylating the threonine residue in a Thr-Pro- or a Thr-Leu-Pro-containing polypeptide. The subject invention also provides a method of determining whether an agent is capable of specifically inhibiting the phosphorylation activity of the ceramide-activated protein kinase. The subject invention further provides a method of determining whether an agent is capable of specifically stimulating the phosphorylation activity of the ceramide-activated protein kinase. The subject invention further provides a method of treating a subject having an inflammatory disorder. The subject invention further provides a method of treating a human subject infected with HIV so as to reduce the proliferation of HIV in the human subject. The subject invention further provides a method of treating a subject having a disorder associated with poor stem cell growth. The subject invention further provides a method of determining whether an agent is capable of specifically inhibiting the ability of lipopolysaccharide to stimulate the phosphorylation activity of the ceramide-activated protein kinase of the subject invention. Finally, the subject invention provides a method of treating a subject suffering from a lipopolysaccharide-related disorder.
3 Claims, 57 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 41

DEPR:
While activation of Raf-1 involves upstream binding to GTP-ras, recent studies have identified the existence of a kinase suppressor of ras (KSR) (201, 202, 203). This putative protein kinase was isolated recently by selection and complementation of genetic mutations in Drosophila and Caenorhabditis elegans (202, 202, 203). KSR appeared to function either upstream of Raf or in parallel with Raf in these systems (204). The predicted size of C. elegans and Drosophila KSR was about 90 and 115 kD, respectively, whereas the size of a murine homolog was about 100 kD. A partial human cDNA has also been sequenced. The N-terminal regions of Drosophila and mammalian KSR contain four conserved domains, CA1-CA4. CA1 is a domain unique to KSR, CA2 is a putative src homology 3 domain, CA3 is a cysteine-rich domain with similarity to the lipid binding moiety of protein kinase C, and CA4 is a serine/threonine-rich domain that resembles the CR2 domain of Raf-1 (201). In all species, the C-terminal region of KSR contains the 11 conserved kinase sub-domains found in all known protein kinases. However, KSR lacks the signature sequences of any specific kinase group, although it is distantly related to the Raf family. KSR is, nonetheless, unlikely to be a Raf family member. The N-terminal ras-binding domain (RBD) which is critical for Raf-ras interaction is absent from KSR. Further, there was no interaction between ras and KSR in the yeast two-hybrid system (201, 203). In addition, kinase subdomain VIII, which is important for substrate recognition, is not conserved between KSR and Raf-1, suggesting that these kinases have different cellular targets. This was confirmed in the yeast two hybrid system which, as predicted, demonstrated strong interaction between Raf-1 and MEK, but not between KSR and MEK (201). Whether KSR might be a tyrosine or serine/threonine kinase is also uncertain. The amino acid sequence YI(L)APE in subdomain VIII of KSR from all species resembles that of a Ser/Thr kinase rather than a tyrosine kinase, which usually contains the consensus

sequence WXAPE. In contrast, both C. elegans and Drosophila KSR contain the HKDLR motif indicative of tyrosine kinases at subdomain VI, while both mammalian KSR possess the HKDLK motif typical of serine/threonine kinases. This implies that the mammalian KSR homologs might represent a distinct subgroup in a KSR superfamily. The mouse and partial human KSR display another interesting feature in kinase subdomain II, in which a conserved lysine residue involved in the phosphotransfer reaction in all mammalian kinases is substituted with arginine. This feature suggests that mammalian KSR might not even function as an active protein kinase. None of the isolated KSR cDNAs have yet been expressed and proven to be active protein kinases.

US-CL-CURRENT: 546/288,546/289 ,546/290 ,546/297 ,546/298

US-PAT-NO: 6022884
DOCUMENT-IDENTIFIER: US 6022884 A
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: February 8, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/352,546/288 ,546/289 ,546/290 ,546/297 ,546/298

ABSTRACT:
Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.
18 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:
The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antagonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of **upstream activators** of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 435/69.1,530/300 ,530/328 ,536/23.5

US-PAT-NO: 5969101
DOCUMENT-IDENTIFIER: US 5969101 A
TITLE: ABL-interactor protein
DATE-ISSUED: October 19, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pendergast; Ann Marie	Durham	NC	N/A	N/A
Dai; Zonghan	Durham	NC	N/A	N/A

US-CL-CURRENT: 530/350,435/69.1 ,530/300 ,530/328 ,536/23.5
ABSTRACT:

The present invention relates to a protein that interacts with the cAbl protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of the invention and cAbl and to the use of such complexes in the identification of therapeutic and diagnostic agents.

13 Claims, 16 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 16

DEPR:
The SH3 domain of the Abi protein of the invention is at the C-terminus (see, for example, aa 346-397 of the Abi protein shown in FIG. 1, A, designated Abi-2). Several proline-rich stretches are present (FIG. 1, B) that constitute binding sites for SH3 domain-containing proteins and contain the consensus PXXP sequence that is present in all high affinity SH3 ligands identified to date (Cohen et al, Cell 80:237 (1995); Rickles et al, EMBO J. 13:5598 (1994)). A polyproline stretch is present upstream of the SH3 domain which could function as a transcriptional activation domain (Tanaka et al, Mol. Cell. Biol. 14:6046 (1994)). Sequences enriched in serine/threonine, glutamate/aspartate and proline residues, designated PEST regions, are also found in the present protein. PEST regions are identified in the central and C-terminal portions of the Abi protein (see FIG. 1, B). The N-terminal region of the present protein is basic (eg, calculated pI about 11.4) and homologous to the DNA-binding sequence of homeodomain proteins (the protein of the invention is unique among the family to which it relates in having both an SH3 domain and a homeodomain homologous region). The C-terminal portion of the protein is acidic (eg, pI about 3.5). A serine-rich region is present in the central portion of the protein (see FIG. 1, B). The Abi protein contains several (eg 9) serine/threonine residues followed by proline, indicative of phosphorylation by proline-directed protein kinases (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Certain sites conform to the cdc 2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse, Cell 61:549 (1990)). There are also potential cAMP-dependent protein kinase sites (eg 11) and potential protein kinase C sites (eg 9) (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Several tyrosines in the sequence are found in peptides that correspond to optimal peptide substrates for the Abl, Fps and Src protein tyrosine kinases (Songyang et al, Nature 373:536 (1995)).

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	3175	src	USPAT; US-PGPUB	2001/05/08 12:13
2	L2	479701	activat$8	USPAT; US-PGPUB	2001/05/08 12:20
3	L3	146498	upstream	USPAT; US-PGPUB	2001/05/08 12:20
4	L4	1449	2 near5 3	USPAT; US-PGPUB	2001/05/08 12:21
5	L5	0	4 near5 1	USPAT; US-PGPUB	2001/05/08 12:22
6	L6	5	4 same 1	USPAT; US-PGPUB	2001/05/08 12:22
7	L7	12	1 same 2 same 3	USPAT; US-PGPUB	2001/05/08 13:57

US-CL-CURRENT: 514/332,514/333 ,546/255 ,546/256 ,546/261 ,546/262 ,546/265 ,546/266

US-PAT-NO: 6184237
DOCUMENT-IDENTIFIER: US 6184237 B1
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: February 6, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/335,514/332 ,514/333 ,546/255 ,546/256 ,546/261 ,546/262 ,546/265 ,546/266

ABSTRACT:
Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, suchas pain and diabetes. The inventin encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermedates useful in such processes.
20 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:
The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducible proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDG, EGF, acidic FDF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antogonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of upstream activators of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 435/320.1,514/2 ,514/44 ,530/300 ,530/350 ,536/23.5 ,536/24.3 ,536/24.31

US-PAT-NO: 6180362
DOCUMENT-IDENTIFIER: US 6180362 B1
TITLE: Peptides which inhibit ras protein activity, their preparation and use
DATE-ISSUED: January 30, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Duchesne; Marc	Sucy-en-Brie	N/A	N/A	FRX
Schweighoffer; Fabien	Vincennes	N/A	N/A	FRX
Tocque; Bruno	Paris	N/A	N/A	FRX

US-CL-CURRENT: 435/69.1,435/320.1 ,514/2 ,514/44 ,530/300 ,530/350 ,536/23.5 ,536/24.3 ,536/24.31

ABSTRACT:
Peptides capable of inhibiting the transforming activity of activated p21 proteins, their preparation and compositions containing them are disclosed. Particular regions of the GAP protein have been identified and characterized that are involved in the transduction of activation signals of p21 proteins. Peptides derived from GAP protein but carrying an effector region rendered non-functional have been shown to inhibit the p21 signaling route. The products according to the invention are useful in pharmaceutical compositions to inhibit the transformation activity of ras genes whose transformation proceeds by a functional p21-GAP interaction.
44 Claims, 3 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 3

BSPR:
More particularly, the invention relates to peptides which are capable of at least partly inhibiting the transformation activity of p21-GTP-GAP complexes. It is furthermore known that p21 proteins are necessary for expression of the transformation power of oncogenes acting upstream, such as src, HER1, HER2 and the like. As a result, the peptides according to the invention and any pharmaceutical composition comprising them can also be used for treatment of tumours having these activated genes.

US-CL-CURRENT: 514/252.05,514/253.01 ,514/256 ,514/269 ,514/272 ,514/274
,514/275 ,514/277 ,514/307 ,514/308 ,514/309 ,514/310 ,514/311 ,514/312
,514/313 ,514/314 ,514/332 ,514/334 ,514/336 ,514/337 ,514/339 ,514/340
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,544/328 ,544/330 ,544/331 ,544/333 ,544/364 ,546/139 ,546/140 ,546/141
,546/142 ,546/143 ,546/144 ,546/148 ,546/152 ,546/153 ,546/155 ,546/156
,546/157 ,546/159 ,546/167 ,546/255 ,546/256 ,546/257 ,546/268.1

US-PAT-NO: 6174901
DOCUMENT-IDENTIFIER: US 6174901 B1
TITLE: Substituted pyridine and pyridazine compounds and methods of use
DATE-ISSUED: January 16, 2001
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Hwang; Chan-Kou	Boulder	CO	N/A	N/A
Spohr; Ulrike D.	Boulder	CO	N/A	N/A

US-CL-CURRENT: 514/333,514/252.05 ,514/253.01 ,514/256 ,514/269 ,514/272
,514/274 ,514/275 ,514/277 ,514/307 ,514/308 ,514/309 ,514/310 ,514/311
,514/312 ,514/313 ,514/314 ,514/332 ,514/334 ,514/336 ,514/337 ,514/339
,514/340 ,514/343 ,544/238 ,544/295 ,544/296 ,544/298 ,544/299 ,544/300
,544/310 ,544/316 ,544/319 ,544/320 ,544/321 ,544/323 ,544/324 ,544/326
,544/327 ,544/328 ,544/330 ,544/331 ,544/333 ,544/364 ,546/139 ,546/140
,546/141 ,546/142 ,546/143 ,546/144 ,546/148 ,546/152 ,546/153 ,546/155
,546/156 ,546/157 ,546/159 ,546/167 ,546/255 ,546/256 ,546/257 ,546/268.1
ABSTRACT:

Selected novel substituted pyridine and pyridazine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as cancer, pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, cancer, pain, diabetes and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.
31 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:
GB 2,306,108, which is incorporated herein by reference in its entirety, describes imidazole derivatives which are Raf kinase antagonists useful in the treatment of cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds may be oncolytics through the antagonism of Raf kinase. It has been reported that antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover, Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of upstream activators of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 435/29

US-PAT-NO: 6117638
DOCUMENT-IDENTIFIER: US 6117638 A
TITLE: Methods to screen for transcription factor-coactivator interactions
DATE-ISSUED: September 12, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kushner; Peter J.	San Francisco	CA	N/A	N/A
Webb; Paul	San Francisco	CA	N/A	N/A
Uht; Rosalie M.	San Francisco	CA	N/A	N/A

US-CL-CURRENT: 435/6,435/29
ABSTRACT:

This invention provides methods for modulating gene expression at the transcriptional level. In particular, the methods involve tethering a transcriptional coactivator to a DNA binding domain that is specific for a target nucleic acid sequence and contacting the coactivator with a transcription factor. The transcription factor triggers or represses transcription mediated by the coactivator. Methods for identifying compounds that are able to modulate gene expression are also provided.
14 Claims, 15 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 10

BSPR:
The mechanism by which estrogen and other modulators of transcription exert their effect on gene expression is complex and incompletely understood. In the case of estrogen and other steroid hormones, for example, the hormone binds to a nuclear receptor that is specific for the hormone. The binding of the hormone to the receptor is believed to cause a conformational change that allows the receptor to bind to certain target sites, often referred to as "response elements," that are located upstream of genes that are regulated by the hormone. Additional proteins are involved in regulating transcription mediated by nuclear hormone receptors. The estrogen receptor (ER), for example, binds two classes of coactivator. The first class, referred to as P160s, includes SRC-1a and GRIP-1/TIF-2. These proteins interact with the ligand binding domain (LBD) of nuclear receptors in a manner that is dependent on hormone and the intactness of the LBD trans-activation function, AF2. Onate et al., Science 270: 1354-7 (1995); Voegel et al., EMBO J. 15: 3667-3675 (1996); Hong et al., Proc. Nat'l. Acad. Sci. USA 93: 4948-52 (1996); Halachmi et al., Science 264: 1455-1458 (1994); Cavailles et al., Proc. Natl. Acad. Sci. USA 91: 10009-10013 (1994). The second class consists of CBP and the closely related protein P300, which are required for transcriptional activation by CREB, Jun/Fos and a growing list of transcription factors. Kamei et al., Cell 85: 403-14 (1996); Chakravarti et al., Nature 383: 99-103 (1996); Smith et al., Proc. Nat'l. Acad. Sci. USA 93: 8884-8888 (1996); Hanstein et al., Proc. Nat'l. Acad. Sci. USA 93: 11540-11545 (1996); Janknecht and Hunter, Nature 383: 22-23 (1996). The two types of coactivator are essential for ER-mediated transcriptional activation. Overexpression of members of the p160 family enhance nuclear receptor action, whereas dominant negative SRC-1a and GRIP-1 block nuclear receptor action. Onate et al., supra.; Hong et al., supra. Similarly, overexpression of CBP, or p300, potentiates ER action, whereas micro-injected antibodies against CBP block nuclear receptor action. Kamei et al., supra.; Chakravarti et al., supra. Since the p160s are tightly bound to CBP and p300 in vivo (Kamei et al., supra.; Hanstein et al., supra.), and SRC-1a and CBP bind to both TBP and TFIIB in vitro (Swope et al., J. Biol. Chem. 271: 28138-28145 (1996); Kwok et al., Nature 370: 223-226 (1994), a model has been hypothesized in which the ER works by recruiting the p160-CBP complex to the promoter, and that this complex forms a bridge to the basal transcription machinery (BTM).

US-CL-CURRENT: 422/68.1,435/283.1 ,435/285.1 ,435/286.1 ,435/286.2 ,435/287.1 ,435/287.2 ,435/287.7 ,435/287.9 ,435/289.1 ,435/299.1

US-PAT-NO: 6077673
DOCUMENT-IDENTIFIER: US 6077673 A
TITLE: Mouse arrays and kits comprising the same
DATE-ISSUED: June 20, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Chenchik; Alex	Palo Alto	CA	N/A	N/A
Lukashev; Matvey	Newton	MA	N/A	N/A

US-CL-CURRENT: 435/6,422/68.1 ,435/283.1 ,435/285.1 ,435/286.1 ,435/286.2 ,435/287.1 ,435/287.2 ,435/287.7 ,435/287.9 ,435/289.1 ,435/299.1
ABSTRACT:

Mouse arrays and methods for their use are provided. The subject arrays include a plurality of polynucleotide spots, each of which is made up of a polynucleotide probe composition of unique polynucleotides corresponding to a key mouse gene. The subject arrays find use in hybridization assays, particularly in assays for the identification of differential gene expression of key mouse genes of interest.

17 Claims, 0 Drawing figures
Exemplary Claim Number: 1

DETL:
type VLA-3 alpha subunit D13867 288-589 NADPH-cytochrome P450 reductase D17571 326-605 Beta-protachykinin a D17584 273-523 Wee1/p87; cdc2 tyrosine 15-kinase D30743 1816-2159 Protein tyrosine phosphatase D83966 1060-1426 Jun-D; c-jun-related transcription J05205 737-964 factor Integrin alpha 7 L23423 2399-2713 Gadd45; growth arrest and DNA- L28177 144-434 damage-inducible protein Bcl-xL apoptosis regulator (bcl-x L35049 641-906 long); Bcl-2 family member N-myc proto-oncogene protein X03919 3262-3450 cAMP-dependent protein kinase type M20473 538-750 I-beta regulatory chain IRF1; interferon regulatory factor 1 M21065 1-233 HSP86; heat shock 86kD protein M36830 255-551 LFA-1 alpha; integrin alpha L; M60778 1838-2050 leukocyte adhesion glycoprotein LFA-1 alpha chain; antigen CD11A (p180) APC; Adenomatous Polyposis Coli M88127 4127-4476 protein Cdc2Sb; cdc2SM2; MPI2 (M-phase S93521 1893-2200 inducer phosphatase 2) P13-K p110; phosphatidylinositol 3- U03279 1437-1723 kinase catalytic subunit RSP27; heat shock 27kD protein 1 U03560 245-500 Csk; c-Src-kinase and negative U05247 645-984 regulator Fasl; Fas antigen ligand; generalized U06948 168-488 lymphoproliferation disease gene (gld) in mice MAPK; MAP kinase; p38 U10871 465-780 p19ink4; cdk4 and cdk6 inhibitor U19597 228-516 Elf-1 Ets family transcription factor U19617 1585-1902 CRAF1; TNF receptor (CD40 U21050 1225-1466 receptor) associated factor; TRAF- related SPI3; serpin; similar to human U25844 915-1230 proteinase inhibitor 6 (placental thrombin inhibitor) serine proteinase inhibitor RIP cell death protein; Fas/APO-1 U25995 1945-2223 (CD95) interactor, contains death domain SLAP; src-like adapter protein; Eck U29056 109-427 receptor tyrosine kinase-associated Atm; ataxia telangiectasia murine U43678 8989-9170 homologue EB1 APC-binding protein U51196 607-834 TANK; I-TRAF; TRAF family U51907 135-437 member associated NF-kB activator Caspase-11; ICH-3 cysteine U59463 352-686 protease; upstream regulator of ICE MLHI DNA mismatch repair U59883 1037-1278 protein; MutL homologue Insulin-like growth factor-IA X04480 183-406 Cell surface glycoprotein MAC-1 X07640 1892-2179 alpha subunit N-ras proto-oncogene; transforming X13664 548-857 G-protein L-myc proto-oncogene protein X13945 5287-5590 CD18 antigen beta subunit X14951 1366-1706 (leukocyte adhesion LFA-1) (CD3, P150, 95) c-Fgr proto-oncogene X52191 1305-1538 Integrin alpha 4 X53176 2176-2449 PKC-beta; protein kinase C beta-II X53532 1712-2089 type HSP60; heat shock 60 kDa protein 1 X53584 1432-1691 (chaperonin, GroEL homologue); mitochondrial matrix protein P1 c-Cbl proto-oncogene (Adaptor X57111 858-1151 protein) Cdc25 phosphatase; guanine X59868 942-1276 nucleotide releasing protein Ezrin; Villin 2; NF-2 (merlin) related X60671 1571-1812 filament/plasma membrane associated protein Cyclin B1 (G2/M-specific) X64713 1184-1447 Integrin alpha 6 X69902 261-611 5-Hydroxytryptamine (serotonin) X72395 1422-1711 receptor 3 Homeobox protein HOXD-3 X73573 141-362 Cyclin E (G1/S-specific) X75888

799-1140 MAPKAPK-2; MAp kinase- X76850 719-987 activated protein kinase; MAPKAP kinase 2 Fra-2 (fos-related antigen 2) X83971 617-844 Cyclin A1 (G2/M-specific) X84311 656-916 DCC; netrin receptor; X85788 4193-4508 immunoglobulin gene superfamily member; former tumor suppressor protein candidate MHR23A; Rad23 UV excision repair X92410 613-955 protein homologue; xerodemia pigmentosum group C (XPC) repair complementing protein MHR23B; Rad23 UV excision repair X92411 542-807 protein homologue; xeroderma pigmentosum group C (XPC) repair complementing protein Integrin beta Y00769 1990-2320 MmRad52; yeast DNA repair protein Z32767 159-417 Rad52 homologue Cyclin G-(G2/M-specific) Z37110 300-619 Prostaglandin E2 receptor EP4 D13458 1146-1442 subtype Interleukin-5 receptor D90205 1389-1739 Epidermal growth factor (EGF) J00380 180-505 Erythropoietin receptor J04843 1193-1377 Insulin receptor J05149 653-1011 p53; tumor suppressor; DNA- K01700 1125-1517 binding protein Cf2r; coagulation factor II L03529 762-1154 (thrombin) receptor PTPRG; protein-tyrosine L09562 1248-1504 phosphatase gamma DNA-binding protein SMBP2 L10075 4790-5088 Interleukin-10 receptor L12120 1762-2110 Interleukin-2 receptor gamma chain L20048 1073-1313 Bone morphogenetic protein 1 L24755 2402-2676 Uromodulin L33406 1809-2136 Thrombopoietin L34169 652-954 Transforming growth factor beta M13177 772-1075 Granulocyte colony- stimulating M13926 86-377 factor (G-CSF) Neuroleukin M14220 1110-1490 Insulin-like growth factor-2 M14951 46-328 (somatomedin A) Interleukin 1 beta M15131 827-1225 c-myb proto-oncogene protein M16449 1212-1513 Tumor necrosis factor beta TNF-beta M16819 461-805 (Lymphotoxin-alpha) Interleukin-1 receptor M20658 2050-2410 CSF-1; M-CSF; colony stimulating X05010 1268-1657 factor-1 Interleukin-4 receptor (membrane- M27959 2469-2705 bound form) Interferon-gamma receptor M28233 1262-1550 Interleukin-7 receptor M29697 701-1104 Gamma interferon induced monokine M34815 42-323 (MIG) Interleukin 10 M37897 175-456 NF-kappa B binding subunit (nuclear M57999 3122-3417 factor) (TFDB5) Tumor necrosis factor receptor 1; M59378 1961-2376 TNFR-1 PDGFRa; platelet-derived growth M84607 474-803 factor alpha-receptor Interleukin-9 receptor M84746 795-1086 iNOS1; nitric oxide synthase M87039 3178-3455 (inducible) Interferon alpha-beta receptor M89641 808-1120 Activating transcription factor 4 M94087 416-769 (mATF4) Beta2-RAR; retinoic acid receptor S56660 589-896 beta-2 Tie-2 proto-oncogene S67051 1843-2179 IGF-I-R alpha; insulin-like growth U00182 489-885 factor I receptor alpha subunit IGFR II; insulin-like growth factor U04710 707-1060

US-CL-CURRENT: 435/15,435/194 ,435/6 ,435/975 ,536/23.2

US-PAT-NO: 6043062
DOCUMENT-IDENTIFIER: US 6043062 A
TITLE: Constitutively active phosphatidylinositol 3-kinase and uses thereof
DATE-ISSUED: March 28, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Klippel; Anke	San Francisco	CA	N/A	N/A
Williams; Lewis T.	Tiburon	CA	N/A	N/A

US-CL-CURRENT: 435/131,435/15 ,435/194 ,435/6 ,435/975 ,536/23.2
ABSTRACT:

The invention provides a method of producing a constitutively active phosphatidylinositol 3-kinase (PI 3-kinase) comprising the catalytic p110 subunit covalently attached at the N-terminus to the iSH2 region of the regulatory subunit, p85. The invention discloses one form of the constitutively active kinase, p110*, which functions independently of growth factor stimulation. Expression vectors encoding a constitutively active PI 3-kinase and cells containing such expression vectors are provided. The invention also provides methods of using the constitutively active phosphatidylinositol 3-kinase to generate phosphoinositides, to identify cellular target proteins and associating molecules of PI 3-kinase, to screen for inhibitors of PI 3-kinase activity and to treat certain diseases, in particular, proliferative diseases. Kits comprising the constitutively active kinase are also provided.

15 Claims, 10 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

BSPR:

Phosphatidylinositol 3-kinase is one of many activities stimulated by growth factors. Phosphatidylinositol 3-kinase is known to be involved in the regulation of cell growth and oncogenic transformation (Cantley et al., Cell, 64:1657 (1993)). The enzyme is found associated with receptor protein tyrosine kinases such as PDGF-R-.beta., CSF-1 receptor, Insulin receptor and IGF-1 receptor as well as non-receptor tyrosine kinase oncogenes, e.g., src, gag-abl and fyn. Studies on mutants of platelet-derived growth factor (PDGF) receptor have shown that phosphatidylinositol 3-kinase is a key mediator of PDGF-mediated mitogenic signaling (Fantl et al., Cell, 69:413 (1992); Valius et al., ibid., 73:321 (1993)). PDGF-R mutants that are unable to bind phosphatidylinositol 3-kinase are also unable to induce a mitogenic response after growth factor stimulation and unable to activate p21.sup.c-ras (ras). These data suggested that phosphatidylinositol 3-kinase acts upstream of ras in PDGF-stimulated signaling.

US-CL-CURRENT: 424/94.5,435/194

US-PAT-NO: 6040149
DOCUMENT-IDENTIFIER: US 6040149 A
TITLE: Assay for identifying agents which act on the ceramide-activated protein kinase, kinase suppressor of ras, and methods of using said agents
DATE-ISSUED: March 21, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kolesnick; Richard N.	New York	NY	N/A	N/A
Liu; Jun	Boston	MA	N/A	N/A
Zhang; Yuhua	New York	NY	N/A	N/A

US-CL-CURRENT: 435/15,424/94.5 ,435/194
ABSTRACT:

The subject invention provides a purified membrane-bound ceramide-activated protein kinase having an apparent molecular weight of about 110 kD as determined by SDS polyacrylamide gel electrophoresis, which protein kinase is capable of specifically phosphorylating the threonine residue in a Thr-Pro- or a Thr-Leu-Pro-containing polypeptide. The subject invention also provides a method of determining whether an agent is capable of specifically inhibiting the phosphorylation activity of the ceramide-activated protein kinase. The subject invention further provides a method of determining whether an agent is capable of specifically stimulating the phosphorylation activity of the ceramide-activated protein kinase. The subject invention further provides a method of treating a subject having an inflammatory disorder. The subject invention further provides a method of treating a human subject infected with HIV so as to reduce the proliferation of HIV in the human subject. The subject invention further provides a method of treating a subject having a disorder associated with poor stem cell growth. The subject invention further provides a method of determining whether an agent is capable of specifically inhibiting the ability of lipopolysaccharide to stimulate the phosphorylation activity of the ceramide-activated protein kinase of the subject invention. Finally, the subject invention provides a method of treating a subject suffering from a lipopolysaccharide-related disorder.
3 Claims; 57 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 41

DEPR:
While activation of Raf-1 involves upstream binding to GTP-ras, recent studies have identified the existence of a kinase suppressor of ras (KSR) (201, 202, 203). This putative protein kinase was isolated recently by selection and complementation of genetic mutations in Drosophila and Caenorhabditis elegans (202, 202, 203). KSR appeared to function either upstream of Raf or in parallel with Raf in these systems (204). The predicted size of C. elegans and Drosophila KSR was about 90 and 115 kD, respectively, whereas the size of a murine homolog was about 100 kD. A partial human cDNA has also been sequenced. The N-terminal regions of Drosophila and mammalian KSR contain four conserved domains, CA1-CA4. CA1 is a domain unique to KSR, CA2 is a putative src homology 3 domain, CA3 is a cysteine-rich domain with similarity to the lipid binding moiety of protein kinase C, and CA4 is a serine/threonine-rich domain that resembles the CR2 domain of Raf-1 (201). In all species, the C-terminal region of KSR contains the 11 conserved kinase sub-domains found in all known protein kinases. However, KSR lacks the signature sequences of any specific kinase group, although it is distantly related to the Raf family. KSR is, nonetheless, unlikely to be a Raf family member. The N-terminal ras-binding domain (RBD) which is critical for Raf-ras interaction is absent from KSR. Further, there was no interaction between ras and KSR in the yeast two-hybrid system (201, 203). In addition, kinase subdomain VIII, which is important for substrate recognition, is not conserved between KSR and Raf-1, suggesting that these kinases have different cellular targets. This was confirmed in the yeast two hybrid system which, as predicted, demonstrated strong interaction between Raf-1 and MEK, but not between KSR and MEK (201). Whether KSR might be a tyrosine or serine/threonine kinase is also uncertain. The amino acid sequence YI(L)APE in subdomain VIII of KSR from all species resembles that of a Ser/Thr kinase rather than a tyrosine kinase, which usually contains the consensus

sequence WXAPE. In contrast, both C. elegans and Drosophila KSR contain the HKDLR motif indicative of tyrosine kinases at subdomain VI, while both mammalian KSR possess the HKDLK motif typical of serine/threonine kinases. This implies that the mammalian KSR homologs might represent a distinct subgroup in a KSR superfamily. The mouse and partial human KSR display another interesting feature in kinase subdomain II, in which a conserved lysine residue involved in the phosphotransfer reaction in all mammalian kinases is substituted with arginine. This feature suggests that mammalian KSR might not even function as an active protein kinase. None of the isolated KSR cDNAs have yet been expressed and proven to be active protein kinases.

DEPR:
The proposed sequence of events suggests an alternative model for Raf-1 **activation**. The well-established paradigm for Raf-1 **activation** through tyrosine kinase receptors, involving adaptor proteins that contain **src** homology domains and the **activation** of ras, does not appear to play a role in TNF-induced Raf-1 **activation**. In this regard, the 55 kD TNF receptor is not a tyrosine kinase and hence would not be expected to link to the set of adaptor proteins promoting ras **activation**. Further, Thr269 comprises a unique recognition site not previously shown to be involved in Raf-1 **activation** by tyrosine kinases. Nevertheless, ras may play a role in TNF-induced Raf-1 **activation**. Preliminary studies show that N17Ras blocked KSR/CAP kinase-induced raf-1 **activation** in COS-7 cells and that electroporation of anti-ras antibody Y13-259 abolished TNF-induced ERK1 **activation** in HL-60 cells. This information is consistent with the genetic data placing KSR downstream of ras. Since KSR/CAP kinase does not bind ras in the yeast two-hybrid assay (201, 203), it is likely that KSR/CAP kinase affects ras action indirectly through its role in modification of raf-1 function. Whether another mechanism exists by which tyrosine kinase receptors can also utilize CAP kinase for signaling through Raf-1 is presently unknown. However, preliminary data show that EGF does not **activate** KSR/CAP kinase in COS-7 cells, and that maximal concentrations of EGF and TNF stimulate more than additive Raf-1 **activation**. These data suggest that TNF and EGF **activate** Raf-1 by different mechanisms. It should be noted that prior studies showed that dominant negative Ras N17 had no effect on TNF-induced stress-**activated** protein kinase (SAPK)/c-Jun kinase (JNK) **activation** in PC12 and COS-7 cells (176; 208). Thus, the **upstream** elements linking TNF to the ERK and SAPK/JNK cascades are likely to be distinct.

US-CL-CURRENT: 546/288,546/289 ,546/290 ,546/297 ,546/298

US-PAT-NO: 6022884
DOCUMENT-IDENTIFIER: US 6022884 A
TITLE: Substituted pyridine compounds and methods of use
DATE-ISSUED: February 8, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mantlo; Nathan B.	Lafayette	CO	N/A	N/A
Schlachter; Stephen T.	Boulder	CO	N/A	N/A
Josey; John A.	Longmont	CO	N/A	N/A

US-CL-CURRENT: 514/352,546/288 ,546/289 ,546/290 ,546/297 ,546/298

ABSTRACT:
Selected novel substituted pyridine compounds are effective for prophylaxis and treatment of diseases, such as TNF-.alpha., IL-1.beta., IL-6 and/or IL-8 mediated diseases, and other maladies, such as pain and diabetes. The invention encompasses novel compounds, analogs, prodrugs and pharmaceutically acceptable salts thereof, pharmaceutical compositions and methods for prophylaxis and treatment of diseases and other maladies or conditions involving inflammation, pain, diabetes, cancer and the like. The subject invention also relates to processes for making such compounds as well as to intermediates useful in such processes.
18 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BSPR:
The present invention also relates to a method of treating cancer which is mediated by Raf and Raf-inducable proteins. Raf proteins are kinases activated in response to extracellular mitogenic stimuli such as PDGF, EGF, acidic FGF, thrombin, insulin or endothelin, and also in response to oncoproteins such as v-src, v-sis, and v-fms. Raf functions downstream of ras in signal transduction from the cellular membrane to the nucleus. Compounds in the present invention may be oncolytics through the antagonism of Raf kinase. Antisense constructs which reduce cellular levels of c-Raf and hence Raf activity inhibit the growth of rodent fibroblasts in soft agar, while exhibiting little or no general cytotoxicity. This inhibition of growth in soft agar is highly predictive of tumor responsiveness in whole animals. Moreover Raf antisense constructs have shown efficacy in reducing tumor burden in animals. Examples of cancers where Raf kinase is implicated by overexpression include cancers of the brain, larynx, lung, lymphatic system, urinary tract and stomach, including hystocytic lymphoma, lung adenocarcinoma and small cell lung cancers. Other examples include cancers involving overexpression of upstream activators of Raf or Raf-activating oncogenes, including pancreatic and breast carcinoma.

US-CL-CURRENT: 435/194,435/21 ,435/252.3 ,435/320.1 ,435/69.1 ,530/300 ,530/350 ,536/23.2

US-PAT-NO: 6001354
DOCUMENT-IDENTIFIER: US 6001354 A
TITLE: GRB2 associating polypeptides and nucleic acids encoding therefor
DATE-ISSUED: December 14, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pot; David A.	San Francisco	CA	N/A	N/A
Williams; Lewis T.	Tiburon	CA	N/A	N/A
Jefferson; Anne Bennett	University City	MI	N/A	N/A
Majerus; Philip W.	University City	MI	N/A	N/A

US-CL-CURRENT: 424/94.5,435/194 ,435/21 ,435/252.3 ,435/320.1 ,435/69.1 ,530/300 ,530/350 ,536/23.2
ABSTRACT:

The present invention generally relates to novel GRB2 associating proteins and nucleic acids which encode these protein. In particular, these novel proteins possess inositol polyphosphate 5-phosphatase and phosphatidylinositol 5-phosphatase activities, important in growth factor mediated signal transduction. As such, the proteins, nucleic acids encoding the proteins, cells capable of expressing these nucleic acids and antibodies specific for these proteins will find a variety of uses in a variety of screening, therapeutic and other applications.

15 Claims, 32 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 27

DRPR:
PtdIns(3,4,5)P.sub.3 in particular, is the product of phosphatidyl inositol 3-kinase ("PI3 kinase"), an important agonist activated signaling protein, stimulated in growth factor mediated signal transduction. PI3-kinase is known to be involved in the regulation of cell growth and oncogenic transformation (Cantley et al., Cell, 64:1657 (1993)). Upon growth factor receptor stimulation, the wild-type PI3-kinase is activated and can phosphorylate phosphatidylinositol ("PtdIns") at the 3' position of the inositol ring. These phosphatidylinositol 3-phosphates are candidate second messenger molecules. The PI3-kinase enzyme is found associated with receptor protein tyrosine kinases such as PDGF-R-.beta., CSF-1 receptor, Insulin receptor and IGF-1 receptor as well as non-receptor tyrosine kinase oncogenes, e.g., src, gag-abl and fyn. Studies on mutants of platelet-derived growth factor (PDGF) receptor have shown that PI3-kinase is a key mediator of PDGF-mediated mitogenic signaling (Fantl et al., Cell, 69:413 (1992); Valius et al., ibid., 73:321 (1993)). PDGF-R mutants that are unable to bind PI3-kinase are also unable to induce a mitogenic response after growth factor stimulation and unable to activate p21c-Ras (Ras). These data indicate that PI3-kinase acts upstream of Ras in PDGF-stimulated signaling. Studies also indicate that the PI3-kinase product, PtdIns(3,4,5)P.sub.3 is not the final product produced during the initial phases of signaling, indicating further processing of this signaling molecule. Stephens, et al., Nature 351:33-39 (1991), Hawkins, et al., Nature 358:157-159 (1992).

US-CL-CURRENT: 435/69.1,530/300 ,530/328 ,536/23.5

US-PAT-NO: 5969101
DOCUMENT-IDENTIFIER: US 5969101 A
TITLE: ABL-interactor protein
DATE-ISSUED: October 19, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Pendergast; Ann Marie	Durham	NC	N/A	N/A
Dai; Zonghan	Durham	NC	N/A	N/A

US-CL-CURRENT: 530/350,435/69.1 ,530/300 ,530/328 ,536/23.5
ABSTRACT:

The present invention relates to a protein that interacts with the cAbl protein tyrosine kinase and to a nucleic acid sequence encoding same. The invention also relates to complexes of the protein of the invention and cAbl and to the use of such complexes in the identification of therapeutic and diagnostic agents.

13 Claims, 16 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 16

DEPR:
The SH3 domain of the Abi protein of the invention is at the C-terminus (see, for example, aa 346-397 of the Abi protein shown in FIG. 1, A, designated Abi-2). Several proline-rich stretches are present (FIG. 1, B) that constitute binding sites for SH3 domain-containing proteins and contain the consensus PXXP sequence that is present in all high affinity SH3 ligands identified to date (Cohen et al, Cell 80:237 (1995); Rickles et al, EMBO J. 13:5598 (1994)). A polyproline stretch is present upstream of the SH3 domain which could function as a transcriptional activation domain (Tanaka et al, Mol. Cell. Biol. 14:6046 (1994)). Sequences enriched in serine/threonine, glutamate/aspartate and proline residues, designated PEST regions, are also found in the present protein. PEST regions are identified in the central and C-terminal portions of the Abi protein (see FIG. 1, B). The N-terminal region of the present protein is basic (eg, calculated pI about 11.4) and homologous to the DNA-binding sequence of homeodomain proteins (the protein of the invention is unique among the family to which it relates in having both an SH3 domain and a homeodomain homologous region). The C-terminal portion of the protein is acidic (eg, pI about 3.5). A serine-rich region is present in the central portion of the protein (see FIG. 1, B). The Abi protein contains several (eg 9) serine/threonine residues followed by proline, indicative of phosphorylation by proline-directed protein kinases (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Certain sites conform to the cdc 2 kinase consensus sequence Ser/Thr-Pro-X-basic (Moreno and Nurse, Cell 61:549 (1990)). There are also potential cAMP-dependent protein kinase sites (eg 11) and potential protein kinase C sites (eg 9) (Kemp and Pearson, Trends Biochem. Sci. 15:342 (1990)). Several tyrosines in the sequence are found in peptides that correspond to optimal peptide substrates for the Abl, Fps and Src protein tyrosine kinases (Songyang et al, Nature 373:536 (1995)).

US-CL-CURRENT: 435/7.1,436/501 ,514/2

US-PAT-NO: 5910417
DOCUMENT-IDENTIFIER: US 5910417 A
TITLE: Regulation of cytokine production in a hematopoietic cell
DATE-ISSUED: June 8, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gelfand; Erwin W.	Englewood	CO	N/A	N/A
Johnson; Gary L.	Boulder	CO	N/A	N/A

US-CL-CURRENT: 435/7.2,435/7.1 ,436/501 ,514/2
ABSTRACT:

A method useful for regulating cytokine production by a hematopoietic cell by regulating an MEKK/JNKK-contingent signal transduction pathway in such a cell is disclosed. Methods of identifying compounds capable of specifically regulating an MEKK/JNKK-contingent signal transduction pathway in hematopoietic cells, a kit for identifying cytokine regulators, methods to treat diseases involving cytokine production, and cells useful in such methods are also set forth.

2 Claims, 28 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 14

DEPR:
The present inventors have unexpectedly found that the PI3-K inhibitor, wortmannin, at concentrations that inhibit PI3-kinase activity, also inhibited JNK activation, but not ERK activation. This finding is the first demonstration of a role for PI3-kinase in regulating a JNK pathway by an Src family tyrosine kinase-associated receptor. Thus, in mast cells the regulation of the MEKK1, JNKK, JNK pathway is dependent on the activation of PI3-kinase, which in turn, is activated by aggregation of FceRI. Mechanistically, there is a very early separation in the signal pathways activated by the Fc.epsilon.RI to differentially regulate JNK and ERK sequential protein kinase pathways. Without being bound by theory, the present inventors believe that PI3-kinase activity is involved in activating the MEKK/JNKK-contigent pathway in mast cells downstream of tyrosine kinases and upstream of MEKK1.

US-CL-CURRENT: 435/320.1,536/23.5

US-PAT-NO: 5541109
DOCUMENT-IDENTIFIER: US 5541109 A
TITLE: Expression cloning of c-src SH3-domain binding proteins
DATE-ISSUED: July 30, 1996
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Searfoss, III; George	Birdsboro	PA	N/A	N/A
H.	Audubon	PA	N/A	N/A
Ivashchenko; Yuri D.	Glenside	PA	N/A	N/A
Jaye; Michael C.	Audubon	PA	N/A	N/A
South; Victoria J.	Phoenixville	PA	N/A	N/A
French; Stephen M.	West Chester	PA	N/A	N/A
Cheadle; Christopher	Blue Bell	PA	N/A	N/A
Ricca; George A.				

US-CL-CURRENT: 435/252.3,435/320.1 ,536/23.5
ABSTRACT:

This invention provides a unique SH3 binding domain core motif of the sequence RPLPXXP and cDNA clones encoding proteins which interact with the SH3 domain of c-src, as well as the amino acid sequences which mediate this binding.

Another embodiment of this invention is a method of identifying SH3-binding proteins and elucidating the sequences which mediate binding. This method may be used as an assay to select compounds which bind to this site and which inhibit or enhance the binding of the SH3 domain.

18 Claims, 30 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 14

DEPR:
In general, L17 bound much less well to the same spectrum of SH3 domains than did L35 or L14, with the notable exception of rasGAP (FIG. 9g), to which only L17 bound weakly and neither L35 nor L14 bound. The reaction of L17 was most strong with the SH3 domains of PI 3-kinase p85a (FIG. 9f) and rasGAP (FIG. 9g). Surprisingly, in this experiment L17 failed to react with the SH3 domain of c-src (FIG. 9a), although L17 was cloned on this basis and bound to the .sup.32 P-GST/PKNSrc SH3 probe but not to the control .sup.32 P-GST/PKA probe (FIGS. 5a,b). The main difference between the experiments presented in FIG. 5 and FIG. 9 is the method of detection of the bound SH3 domains: in FIG. 5 the GST/PKNSrc SH3 fusion protein was phosphorylated in vitro with .sup.32 P-g-ATP and detected by autoradiography, whereas in FIG. 9 bound GST/SH3 fusion proteins were detected with anti GST antibodies followed by ECL. These results suggested that serine phosphorylation of the PKA recognition site within the GST/PKNSrc SH3 fusion protein was crucial for the binding of of the src SH3 sequence to the L17 protein. To test this possibility, the GST/PKNSrc SH3 fusion protein was phosphorylated in vitro with non-radioactive ATP, reacted with filters containing the L14, L17 and L35 proteins, and detected with anti-GST antibodies and ECL. Indeed, the presence of the phosphorylated serine in the c-srcSH3 domain's amino terminal flanking sequence was crucial for the binding to the L17 fusion protein (FIGS. 9a,b,e). A fusion protein in which the PKA recognition site was situated downstream of the c-srcSH3 domain and phosphorylated in vitro with .sup.32 P-.gamma.-ATP was unable to bind L17, but bound to L35 and L14 like the original .sup.32 P-GST/PKNSrc SH3 probe (data not shown). These results argue that it is not the phosphorylated PKA site within the GST/PKNSrc SH3 fusion protein per se which is responsible for the interaction with L17. Rather, these results demonstrate that serine phosphorylation within the flanking sequence amino terminal to the c-srcSH3 domain is necessary for the recognition of L17 by the GST/PKNSrc SH3 fusion protein. Consistent with these findings, only L14 and L35 proteins but not L17 were able to bind to full length autophosphorylated c-src kinase in vitro. It is uncertain whether in vivo the affinity or specificity of binding of the c-srcSH3 domain is similarly regulated by upstream serine/threonine phosphorylation. This may also be applicable for the SH3 binding motifs. In such cases, phosphorylation-dephosphorylation may potentially serve as an

important regulator of SH3 domain binding. The importance of serine/threonine phosphorylation in controlling the growth factor activated MAP kinase pathway was demonstrated recently (49, 50). In these experiments, the association of raf-1 kinase with p21 ras was inhibited by phosphorylation of raf-1 on serine 43 by the cAMP-dependent protein kinase, PKA. In a similar manner, cAMP-elevating agents which activate PKA may potentially regulate interactions between SH3 domains and SH3 binding proteins. We have found that the SH3-binding protein L14 can be phosphorylated by src kinase in vitro, and several candidate tyrosine phosphorylation sites are indicated in the L14 sequence (FIG. 6). In this respect, L14 protein resembles actin filament-associated protein AFAP-110 which has srcSH3 binding sites (FIG. 10) and is tyrosine phosphorylated by src kinase (51). Whether or not this phosphorylation is physiologically important is unclear.

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	3175	src	USPAT; US-PGPUB	2001/05/08 12:13
2	L2	479701	activat$8	USPAT; US-PGPUB	2001/05/08 12:20
3	L3	146498	upstream	USPAT; US-PGPUB	2001/05/08 12:20
4	L4	1449	2 near5 3	USPAT; US-PGPUB	2001/05/08 12:21
5	L5	0	4 near5 1	USPAT; US-PGPUB	2001/05/08 12:22
6	L6	5	4 same 1	USPAT; US-PGPUB	2001/05/08 12:22
7	L7	12	1 same 2 same 3	USPAT; US-PGPUB	2001/05/08 13:57
8	L8	133	1 near2 2	USPAT; US-PGPUB	2001/05/08 14:25
9	L9	72	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2001/05/08 14:37
10	L10	1538	hbv or hbx	USPAT; US-PGPUB	2001/05/08 14:37
11	L11	1	9 and 10	USPAT; US-PGPUB	2001/05/08 14:37

US-CL-CURRENT: 435/320.1,536/24.1

US-PAT-NO: 5885833
DOCUMENT-IDENTIFIER: US 5885833 A
TITLE: Nucleic acid constructs for the cell cycle-regulated expression of genes and therapeutic methods utilizing such constructs
DATE-ISSUED: March 23, 1999
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Mueller; Rolf	Marburg	N/A	N/A	DEX
Zwicker; Joerk	Marburg	N/A	N/A	DEX
Sedlacek; Hans-Harald	Marburg	N/A	N/A	DEX

US-CL-CURRENT: 435/372,435/320.1 ,536/24.1
ABSTRACT:
Nucleic acid constructs comprising an activator sequence, a promoter module, and a structural gene are disclosed. The promoter module comprises a a CHR region and a nucleic acid sequence that binds a protein of the E2F family. These constructs are used in gene therapy, such as the treatment of disorders characterized by excess cell proliferation.
19 Claims, 7 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

BSPR:
In a further embodiment, the invention relates to a nucleic acid construct as described above, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses HBV, HCV, HSV, HPV, EBV, HTLV, CMV, SV40 or HIV.

DEPR:
In one embodiment, the activator sequence (a) in the nucleic acid constructs according to the invention is cell-specific or virus-specific or metabolic specific. As used in this specification, "cell-specific" means that the activator sequence is selected from a gene coding for a protein that is specifically expressed in a given cell, and "virus-specific" means that the activator sequence is selected from a viral gene; metabolic specific means, that the activator sequence is selected from a gene, coding for a protein, that is specifically expressed under defined metabolic conditions. Thus in another embodiment, the nucleic acid constructs according to the invention have an activating sequence (a) which is selected from the group of promoters or enhancers which activate transcription in endothelial cells, smooth muscle cells, hemopoietic cells, lymphocytes, macrophages, tumor cells, leukemia cells or glial cells, or from promoter sequences or enhancer sequences of the viruses HBV, HCV, HSV, HPV, EBV, HTLV or HIV. Examples of cell-specific activator sequences, virus-specific sequences and metabolic specific sequences are described below.

DEPR:
The activator sequence to be chosen comprises promoter sequences from cellular genes whose activity is altered in particular by infections with bacteria or parasites, or the promoter sequences to be chosen are those from viruses which transform the cells infected by them and stimulate proliferation. These viruses include, for example, HBV, HCV, HSV, HPV, HIV, EBV and HTLV.

CLPR:
16. A nucleic acid construct as claimed in claim 1, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses HBV, HCV, HSV, HPV, EBV, HTLV, CMV, SV40 and HIV.

ORPL:
Mukhopadnyay et al., "Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation," Nature 375, (1995), pp. 577-581.

	L #	Hits	Search Text	DBs	Time Stamp
1	L1	3175	src	USPAT; US-PGPUB	2001/05/08 12:13
2	L2	479701	activat$8	USPAT; US-PGPUB	2001/05/08 12:20
3	L3	146498	upstream	USPAT; US-PGPUB	2001/05/08 12:20
4	L4	1449	2 near5 3	USPAT; US-PGPUB	2001/05/08 12:21
5	L5	0	4 near5 1	USPAT; US-PGPUB	2001/05/08 12:22
6	L6	5	4 same 1	USPAT; US-PGPUB	2001/05/08 12:22
7	L7	12	1 same 2 same 3	USPAT; US-PGPUB	2001/05/08 13:57
8	L8	133	1 near2 2	USPAT; US-PGPUB	2001/05/08 14:39
9	L9	72	1 near2 (activator$1 or activation)	USPAT; US-PGPUB	2001/05/08 14:37
10	L10	1538	hbv or hbx	USPAT; US-PGPUB	2001/05/08 14:37
11	L11	1	9 and 10	USPAT; US-PGPUB	2001/05/08 14:39
12	L12	201	1 near5 2	USPAT; US-PGPUB	2001/05/08 14:39
13	L13	7	10 and 12	USPAT; US-PGPUB	2001/05/08 14:39

US-CL-CURRENT: 530/300,530/350

US-PAT-NO: 6030822
DOCUMENT-IDENTIFIER: US 6030822 A
TITLE: Extracellular signal-regulated kinase, sequences, and methods of production and use
DATE-ISSUED: February 29, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lechner; Cornelia	Unterschleissheim	N/A	N/A	DEX
M.o slashed.ller; Niels	Copenhagen	N/A	N/A	DKX
Peter	Martinsreid	N/A	N/A	DEX
Ullrich; Axel				

US-CL-CURRENT: 435/194,530/300 ,530/350
ABSTRACT:

The present invention relates, in general, to an extracellular signal regulated kinase, ERK-5. In particular, the present invention relates to nucleic acid molecules coding for ERK-5; ERK-5 polypeptides; recombinant nucleic acid molecules; cells containing the recombinant nucleic acid molecules; antisense ERK-5 nucleic acid constructs; antibodies having binding affinity to an ERK-5 polypeptide; hybridomes containing the antibodies; nucleic acid probes for the detecting of ERK-5 nucleic acid; a method of detecting ERK-5 nucleic acid or polypeptide in a sample; kits containing nucleic acid probes or antibodies; a method of detecting a compound capable of binding to ERK-5 or a fragment thereof; a method of detecting an agonist or antagonist of ERK-5 activity; a method of agonizing or antagonizing ERK-5 associated activity in a mammal; a method of treating diabetes mellitus, skeletal muscle diseases, Alzheimer's disease, or peripheral neuropathies in a mammal with an agonist or antagonist of ERK-5 activity; and a pharmaceutical composition comprising an ERK-5 agonist or antagonist.

6 Claims, 2 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 5

BSPR:
While some of these muscle specific transcription factors, namely MyoD and myf5, are constitutively expressed both in cycling myoblasts as well as in myotubes, myogenin expression is induced when myoblasts start to differentiate. However, not only transcriptional regulation, but also posttranslational modification such as phosphorylation which has been reported for MyoD1 and myogenin as well as myf5 may influence commitment to myogenesis and maintenance of the differentiated state. **Activated oncogenes like ras and src** as well as growth factors which are involved in or initiate signal transduction, inhibit myogenesis. In addition, PKC is able to phosphorylate myogenin and could be a major mediator of this inhibition.

DEPR:
NIH3T3 cells, immortalized mouse fibroblasts (Jainchill et al., J. Virol. 4:549-553 (1969)) were grown in DMEM with 4.5 mg/ml glucose and 10% FCS to subconfluency and transfected with 20 .mu.g/1.times.10.sup.7 cells of a cvn-construct containing the complete hERK-5 cDNA. The cvn vector carries the SV40 early promoter, **HBV** poly A signal as well as a neomycin resistance gene which allows selection of transfected cells on G418 resistance, and the gene for the DHFR which can be used to increase the expression of the integrated cDNA by addition of methotrexate at concentrations of 100-1000 nM to the culture medium (Rosenthal et al., Cell 46:155-169 (1986)). Transfection was performed as described in Example 4. After 16 h at 35.degree. C. and 3% CO.sub.2, the medium was changed and the cells were grown at 37.degree. C. 5% CO.sub.2 for additional 24 h with one medium change after 8 h. The cells were then split to different dilutions and grown in 1 mg/ml G418 containing medium until cell colonies appeared which were isolated and selected on methotrexate growth.

US-CL-CURRENT: 435/320.1,435/325 ,435/410 ,435/455 ,435/456 ,536/23.5 ,536/23.72 ,536/24.1

US-PAT-NO: 6015686
DOCUMENT-IDENTIFIER: US 6015686 A
TITLE: Eukaryotic layered vector initiation systems
DATE-ISSUED: January 18, 2000
INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 435/69.1,435/320.1 ,435/325 ,435/410 ,435/455 ,435/456 ,536/23.5 ,536/23.72 ,536/24.1
ABSTRACT:
The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.
20 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the

antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8.sup.+ T cells, such that the CD8.sup.+ T cells produce enough IL-2 to expand and become fully activated. These CD8.sup.+ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8.sup.+ T cell via the costimulatory ligand B7/BB1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 1981; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the

hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

DEPR:
Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of .beta.-galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m.

injection. Mice injected with ELVIS-.beta.-gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135, 1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
1. Site-Directed Mutagenesis of HBV E/Core Sequence Utilizing PCR

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop

codon of the HBV precore/core coding region.

DEPR:
The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
2. Isolation of HBV Core Sequence

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK.sup.+ HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK.sup.+ HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3 M sodium acetate is added to this solution followed by 500 .mu.l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu.l deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK.sup.+ plasmid. This plasmid is designated SK+HBc.

DEPR:
3. Isolation of HBV X Antigen

DEPR:
4. Construction of Sindbis Vectors Expressing HBVE, HBV Core and HBV X

DEPR:
Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:

Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSIN-HBx.

DEPR:
The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/ml G418. The Jurkat A2/K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or

HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5 .mu.Ci .sup.3 H-thymidine is added to the CTLL-2 cells. 0.5 Ci .sup.3 H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

DEPR:
1. Construction of ELVIS Vectors Expressing HBVE-C, HBV Core and HBV X

DEPR:
Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK.sup.+ HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK.sup.+ HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Bio101). Sindbis vector plasmid pKSSINd1JRsjrc (Example 3) also is digested with ApaI, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP,

purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepal-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSd1-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Addtional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

DEPL:
A. Isolation of HBV E/Core Sequence

DEPL:
G. Generation of ELVIS Vector Constructs Which Express HBV Antigens for the Induction of an Immune Response

DEPC:
Generation of Vector Constructs Which Express HBV Antigens for the Induction of an Immune Response

CLPR:
9. The eukaryotic layered vector initiation system according to claim 1, wherein said heterologous sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and CMV.

CLPR:
11. The eukaryotic layered vector initiation system according to claim 10, wherein said antisense sequence or non-coding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, and CMV sequences.

US-CL-CURRENT: 424/199.1,424/204.1 ,424/228.1 ,424/234.1 ,424/265.1 ,424/274.1 ,424/277.1 ,536/23.5 ,536/23.7 ,536/23.72

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INVENTOR-INFORMATION:

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ABSTRACT:
The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.
11 Claims, 35 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells

requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB 1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB 1 may be introduced into tumor cells in order to cause costimulation of CD8+T cells, such that the CD8+T cells produce enough IL-2 to expand and become fully activated. These CD8+T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic **HBV** core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The **HBV** core-specific CTL response will be augmented from the fully activated CD8+T cell via the costimulatory ligand B7/BB 1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "**HBV**" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 198 1; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an **HBx** antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of **HBV**, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the

hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

DEPR:
Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF S and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF S and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of -galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m. injection.

Mice injected with ELVIS-.beta.-gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p005 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
1. Site-Directed Mutagenesis of HBV E/Core Sequence Utilizing PCR

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop

codon of the HBV precore/core coding region.

DEPR:
The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
2. Isolation of HBV Core Sequence

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK+HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK+HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 rl ricrofuge tube. Fifty microliters of 3 M sodium acetate is added to this solution followed by 500 .mu.l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu.l deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK+plasmid. This plasmid is designated SK+HBc.

DEPR:
3. Isolation of HBV X Antigen

DEPR:
4. Construction Of Sindbis Vectors Expressing HBVe, HBV Core and HBV X

DEPR:
Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:

Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSIN-HBx.

DEPR:
The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CGL targets in the following sections.

DEPR:
Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK.sup.-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.
2. I OPRECIPITATIONIWETERN BLOT

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/ml G418. The Jurkat A2/K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat

96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CIL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5.mu. Ci.sup.3 H-thymidine is added to the CTLL-2 cells. 0.5Ci .sup.3 H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Manrheim (Indianapolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium.release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CIls in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, EL) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as

in Example 13E 1.c.

DEPR:
1. Construction of Elvis Vectors Expressing HBVe-c. HBV Core and HBV X

DEPR:
Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK+HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK+HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SMBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CIL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-E antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISBBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balblc, C57B 16, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Biol01). Sindbis vector plasmid pKSSINdlJRsjrc (Example 3) also is digested with ApaI, under limited conditions

to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Addtional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

DEPL:
A. Isolation of HBV E/Core Sequence

DEPL:
G. Generation of Elvis Vector Constructs Which Express HBV Antigens for the Induction of an Immune Response

DEPC:
Generation of Vector Constructs Which Express HBV Antigens for the Induction of an Immune Response

CLPR:
3. The method according to claim 2 wherein said viral antigen is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-1, HTLV-2, and CMV.

US-CL-CURRENT: 435/320.1,536/24.1

US-PAT-NO: 5885833
DOCUMENT-IDENTIFIER: US 5885833 A
TITLE: Nucleic acid constructs for the cell cycle-regulated expression of genes and therapeutic methods utilizing such constructs
DATE-ISSUED: March 23, 1999
INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/372,435/320.1 ,536/24.1
ABSTRACT:
Nucleic acid constructs comprising an activator sequence, a promoter module, and a structural gene are disclosed. The promoter module comprises a a CHR region and a nucleic acid sequence that binds a protein of the E2F family. These constructs are used in gene therapy, such as the treatment of disorders characterized by excess cell proliferation.
19 Claims, 7 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

BSPR:
In a further embodiment, the invention relates to a nucleic acid construct as described above, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses **HBV,** HCV, HSV, HPV, EBV, HTLV, CMV, SV40 or HIV.

DEPR:
In one embodiment, the activator sequence (a) in the nucleic acid constructs according to the invention is cell-specific or virus-specific or metabolic specific. As used in this specification, "cell-specific" means that the activator sequence is selected from a gene coding for a protein that is specifically expressed in a given cell, and "virus-specific" means that the activator sequence is selected from a viral gene; metabolic specific means, that the activator sequence is selected from a gene, coding for a protein, that is specifically expressed under defined metabolic conditions. Thus in another embodiment, the nucleic acid constructs according to the invention have an activating sequence (a) which is selected from the group of promoters or enhancers which activate transcription in endothelial cells, smooth muscle cells, hemopoietic cells, lymphocytes, macrophages, tumor cells, leukemia cells or glial cells, or from promoter sequences or enhancer sequences of the viruses HBV, HCV, HSV, HPV, EBV, HTLV or HIV. Examples of cell-specific activator sequences, virus-specific sequences and metabolic specific sequences are described below.

DEPR:
The activator sequence to be chosen comprises promoter sequences from cellular genes whose activity is altered in particular by infections with bacteria or parasites, or the promoter sequences to be chosen are those from viruses which transform the cells infected by them and stimulate proliferation. These viruses include, for example, **HBV,** HCV, HSV, HPV, HIV, EBV and HTLV.

CLPR:
16. A nucleic acid construct as claimed in claim 1, wherein said activator sequence (a) is a promoter or enhancer sequence from a virus, wherein said virus is selected from the group consisting of the viruses **HBV,** HCV, HSV, HPV, EBV, HTLV, CMV, SV40 and HIV.

ORPL:
Mukhopadnyay et al., "Hypoxic induction of human vascular endothelial growth factor expression through c-**Src activation,**" Nature 375, (1995), pp. 577-581.

US-CL-CURRENT: 435/235.1,435/320.1 ,435/325

US-PAT-NO: 5843723
DOCUMENT-IDENTIFIER: US 5843723 A
TITLE: Alphavirus vector constructs
DATE-ISSUED: December 1, 1998
INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/69.3,435/235.1 ,435/320.1 ,435/325
ABSTRACT:
The present invention provides compositions and method,, for utilizing recombinant alphavirus vectors.
47 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the

antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8.sup.+ T cells, such that the CD8.sup.+ T cells produce enough IL-2 to expand and become fully activated. These CD8.sup.+ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8.sup.+ T cell via the costimulatory ligand B7/BB1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 1981; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant ".alpha.". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two

areas of the hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

DEPR:
Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of .beta.-galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m.

injection. Mice injected with ELVIS-.beta.-gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:

The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK+HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 31 end that was present in the multicloning site of SK+HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3M sodium acetate is added to this solution followed by 500 .mu.l of chloroform: isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu.l deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK.sup.+ plasmid. This plasmid is designated SK+HBc.

DEPR:
Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:
Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSIN-HBx.

DEPR:
The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to

coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/ml G418. The Jurkat A2/K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro.

Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5 .mu.Ci .sup.3 H-thymidine is added to the CTLL-2 cells. 0.5Ci .sup.3 H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

DEPR:
Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK.sup.+ HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and

ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK.sup.+ HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard,

DEPR:
Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
A. ISOLATION OF HBV E/CORE SEQUENCE

DEPL:
1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

DEPL:
2. ISOLATION OF HBV CORE SEQUENCE

DEPL:
3. ISOLATION OF HBV X ANTIGEN

DEPL:
4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBVE, HBV CORE AND HBV X

DEPL:
G. GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE

INDUCTION OF AN IMMUNE RESPONSE

DEPL:
1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBVE-C. HBV CORE AND HBV X

DEPC:
GENERATION OF VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

DEPV:
5'-TATATGGGCCCGTACGGAAGGAAAGAAGTCA-3' (SEQ.ID NO. 118) Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Bio101). Sindbis vector plasmid pKSSINdlJRsjrc (Example 3) also is digested with ApaI, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

DEPV:
5'-TATATGGGCCCATCGAGGTGAGAAAGAGGAC-3' (SEQ. ID NO. 125) Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Additional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

CLPR:
13. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and CMV.

US-CL-CURRENT: 435/320.1,536/23.1 ,536/24.1

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INVENTOR-INFORMATION:

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ABSTRACT:
The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.
25 Claims, 37 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to

as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB 1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8.sup.+ T cells, such that the CD8.sup.+ T cells produce enough IL-2 to expand and become fully activated. These CD8.sup.+ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB 1 factor and, for example, an immunogenic **HBV** core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The **HBV** core-specific CTIL response will be augmented from the fully activated CD8.sup.+ T cell via the costimulatory ligand B7/BB1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Innmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "**HBV**" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 1981; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an **HBx** antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of **HBV**, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open

reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

DEPR:
Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of .beta.-galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m. injection. Mice injected with ELVIS-.beta.-gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which

were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and **activates the membrane tyrosine kinase, src,** and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCAvwhich occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
2. ISOLATION OF HBV CORE SEQUENCE

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK+HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK.sup.+ HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3M sodium acetate is added to this solution followed by 500 .mu.l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu. deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK.sup.+ plasmid. This plasmid is designated SK+HBc.

DEPR:
3. ISOLATION OF HBV X ANTIGEN

DEPR:
4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBVE, HBV CORE AND HBV X

DEPR:
Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:
Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a

cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSIN-HBx.

DEPR:
The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/rml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/mml G418. The Jurkat A.sub.2 /K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of

rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5.mu. Ci .sup.3 H-thymidine is added to the CTLL-2 cells. 0.5Ci .sup.3 H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

DEPR:

1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBVE-C. HBV CORE AND HBV X

DEPR:
Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK.sup.+ HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK.sup.+ HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Biol01). Sindbis vector plasmid pKSSINd1JRsjrc (Example 3) also is digested with ApaI, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus

vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Additional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

DEPL:
A. ISOLATION OF HBV E/CORE SEQUENCE

DEPL:
G . GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

DEPC:
GENERATION OF VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

CLPR:
11. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from HBV.

CLPR:
23. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HBV.

US-CL-CURRENT: 435/325,435/69.1 ,536/23.72

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DOCUMENT-IDENTIFIER: US 5789245 A
TITLE: Alphavirus structural protein expression cassettes
DATE-ISSUED: August 4, 1998
INVENTOR-INFORMATION:

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US-CL-CURRENT: 435/320.1,435/325 ,435/69.1 ,536/23.72
ABSTRACT:
The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.
29 Claims, 35 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 30

BSPR:
In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

BSPR:
The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

BSPR:
Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

DEPR:
Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon (.gamma.-IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

DEPR:
Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the

antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., J. Exp. Med., 173:721-730, 1991a, and J. Exp. Med., 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8.sup.+ T cells, such that the CD8.sup.+ T cells produce enough IL-2 to expand and become fully activated. These CD8.sup.+ T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8.sup.+ T cell via the costimulatory ligand B7/BB1.

DEPR:
Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., E. coli, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immmunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC Class II presentation, or both.

DEPR:
Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., Science 213:406-411, 1981; Tiollais et al., Nature 317:489-495, 1985; and Ganem and Varmus, Ann. Rev. Biochem. 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., TIG 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

DEPR:
As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, Progr. Allergy 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, J. Infect. 123:671, 1971; Bancroft et al., J. Immunol. 109:842, 1972; and Courouce et al., Bibl. Haematol. 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the

hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw.sub.2 is more abundant (Molecular Biology of the Hepatitis B Virus, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

DEPR:
Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., Hepatology 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

DEPR:
A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., Nature 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, Biotechniques 6:616-627, 1988; Rosenfeld et al., Science 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., J. Vir. 63:3822-3828, 1989; Mendelson et al., Virol. 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, Adv. Exp. Med. Biol. 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, J. Virol. 65:532-536, 1991).

DEPR:
Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DEPR:
Using techniques described above, the lacZ gene encoding the .beta.-galactosidase reporter protein was cut from the plasmid pSV-.beta.-galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS-.beta.-gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of .beta.-galactocidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m.

injection. Mice injected with ELVIS-.beta.-gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

DEPR:
In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33.sup.cdk2 and p34.sup.cdc2. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., Cell 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., Cancer Research 54:3131-3135,1994).

DEPR:
In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. EMBO J. 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. EMBO J. 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking ApaI recognition sequences:

DEPR:
In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the altenative approaches described in Example 8.

DEPR:
1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop

codon of the HBV precore/core coding region.

DEPR:
The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

DEPR:
The first PCR reaction corrects the deletion in the antisense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

DEPR:
The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

DEPR:
To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

DEPR:
The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK.sup.+ HBe plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK.sup.+ HBe plasmid.

DEPR:
Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3M sodium acetate is added to this solution followed by 500 .mu.l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20.degree. C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500 .mu.l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 .mu.l deionized H.sub.2 O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK.sup.+ plasmid. This plasmid is designated SK+HBc.

DEPR:
Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN.TM. and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

DEPR:
Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSIN-HBx.

DEPR:
The above Sindbis HBV expressing vectors may also be modified to coexpress a

selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0.times.10.sup.7 cultured cells with PBS, resuspending the cells to a total volume of 600 .mu.l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

DEPR:
As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

DEPR:
As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from 10.sup.6 L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

DEPR:
Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

DEPR:
Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriaste cell line The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800 .mu.g/ml G418. The Jurkat A2/K.sup.b cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

DEPR:
Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 .mu.g/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

DEPR:
Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37.degree. C. and 5% CO.sub.2 in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10.sup.-5 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37.degree. C. and 5%, CO.sub.2 for 3 days. Subsequently, 0.5.mu. Ci .sup.3 H-thymidine is added to the CTLL-2 cells. 0.5Ci .sup.3 H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

DEPR:
The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

DEPR:
The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10.sup.7 or 10.sup.8 pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

DEPR:
Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

DEPR:
Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK.sup.+ HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

DEPR:
The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and ligated into SK+II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK+HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK+HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN.TM., and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

DEPR:
Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

DEPR:
Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

DEPR:
The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE.TM. and transfected into 2.times.10.sup.5 BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

DEPR:
As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

DEPR:
The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI and purified from a 1.5% agarose gel using MERMAID.TM. (Bio101). Sindbis vector plasmid pKSSINdlJRsjrc (Example 3) also is digested with ApaI, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., EMBO J. 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepal-6 (ATCC #CRL1830), and selected using the

drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

DEPL:
Following amplification, the PCR amplicon is digested with ApaI, purified from a 1.5% agarose gel using MERMAID.TM., and ligated into pKSSINdlJRsjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., NAR 11:883, 1983); membrane (M protein, Armstrong et al., Nature 308:751, 1984); and spike (S protein, Luytjes et al., Virology 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVSdl-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Addtional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

DEPL:
A. ISOLATION OF HBV E/CORE SEQUENCE

DEPL:
2. ISOLATION OF HBV CORE SEQUENCE

DEPL:
3. ISOLATION OF HBV X ANTIGEN

DEPL:
4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBV E, HBV CORE AND HBV X

DEPL:
G. GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

DEPL:
1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBV E- C, HBV CORE AND HBV X

DEPC:
GENERATION OF VECTOR CONSTRUCTS WHICH EXPRESS HBV A NTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE